4′-Methylangelicins: New Potential Agents for the Photochemotherapy of Psoriasis

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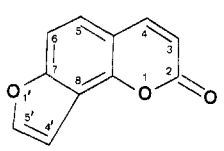
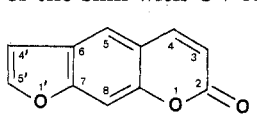
Three derivatives of angelicin (1) [4′-methyl-, 4,4′-dimethyl-, and 4′,5-dimethylangelicin (**2a–c**)] have been prepared with the aim of obtaining new agents for the photochemotherapy of psoriasis. These compounds form a complex in the dark with DNA that shows an affinity for the macromolecule higher than that of the parent angelicin (1). A correlation between their octanol/water partition coefficients and the association constants of the complexes has been observed. Compounds **2a–c** photobind to DNA to a much higher extent than 1 and also more effectively than 8-methoxypsoralen (8-MOP), taken as reference compound. When activated with UV-A, the three compounds strongly inactivate T_2 phage and inhibit epidermal DNA synthesis in mice. Moreover, they show a mutagenic activity markedly lower than that of 8-methoxypsoralen on *Escherichia coli* wild-type strain. Due to its lack of skin phototoxicity, its low mutagenic activity, and its antiproliferative activity, **2c** was chosen for clinical evaluation. It proved to be effective in clearing psoriasis in two patients.

PUVA therapy (psoralen plus UV-A light), a particular type of chemotherapy for proliferative skin diseases (psoriasis, mycosis fungoides, and others) is realized by oral or topical administration of a linear furocoumarin (psoralen) and successive irradiation of the skin with UV-A



angelicin (1)



psoralen

light.[1-4] The antiproliferative effect of this treatment is due to the photoinduction by psoralen of selective monofunctional and bifunctional lesions (interstrand crosslinkages) to the DNA of the skin cell.[1] This treatment appears the most effective for some kinds of psoriasis and for the early stages of mycosis fungoides; however, side effects, such as skin phototoxicity,[1-4] a certain, although low, risk of skin cancer,[5] and, when psoralen is administered orally, a potential risk of cataracts[4,6] and of hepatotoxicity,[7] have been reported. Recently, with the aim of eliminating skin phototoxicity or reducing the risk of skin cancer, we have prepared some monofunctional furocoumarins (methylangelicins) that have proved to be capable of realizing their antiproliferative and therapeutical activity by inducing only monofunctional photolesions to DNA, maintaining, however, the same selectivity of action as psoralens.[8-11] Continuing this line of research, we describe in this paper the preparation of a series of 4′-methylangelicins [4′-methylangelicin (**2a**), 4′,4-dimethylangelicin (**2b**), and 4′,5-dimethylangelicin (**2c**)], their interactions with DNA, their antiproliferative activity, and some preliminary clinical evaluations.

Results

Chemistry. A procedure previously described[12,13] has been followed for the preparation of **2a–c**. The Fries rearrangement was carried out on the starting products, i.e., acetylated umbelliferone (7-acetoxycoumarin, **3a**), its 4-methyl derivative (**3b**), or its 5-methyl derivative (**3c**) (see Scheme I). By this reaction the acetyl group underwent migration mainly to the 8-position, yielding the 8-acetylumbelliferones (**4a–c**); to a much lesser extent, it also underwent migration to the 6-position, forming the corresponding 6-acetyl isomers (**5a–c**). At this level, an accurate chromatographic separation of the 8-acetyl from the

Scheme I

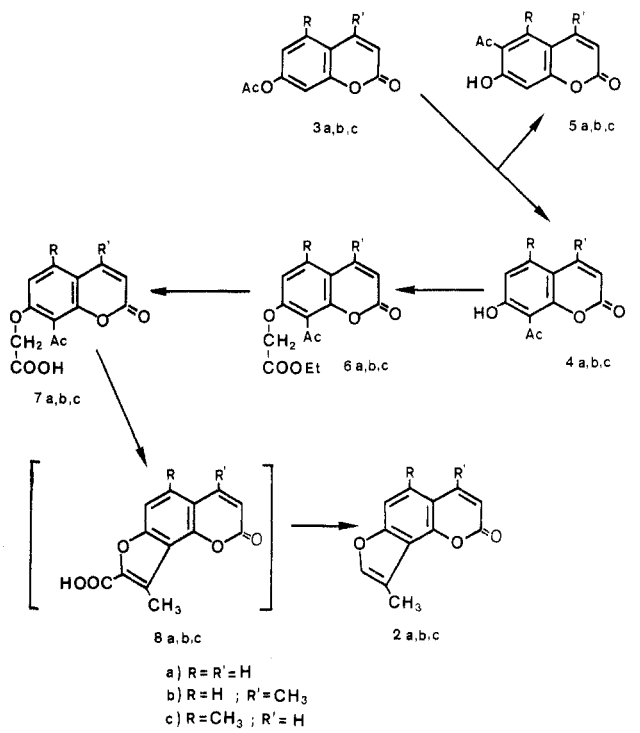


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Table I. Water Solubilities and Octanol/Water Partition Coefficients of the Compounds and Binding Parameters of Their Complexes with Calf Thymus DNA

compd	water solubility		partition coefficient (octanol/water)	K	n[a]	$1/n$[b]
	μg/mL	mol/L				
1[c]	20	10.5×10^{-5}	120	560	15.87	0.063
2a	14	7.0×10^{-5}	240	975 ± 88	15.38 ± 0.15	0.065
2b	2.6	1.2×10^{-5}	1190	6600 ± 354	12.50 ± 0.12	0.080
2c	4.7	2.2×10^{-5}	1041	5400 ± 625	12.50 ± 0.12	0.080
8-MOP[d]	23.0	10.6×10^{-5}		736	7.81	0.128

[a] According to Mc Ghee and von Hippel,[18] n is defined here as the number of nucleotides occluded by one molecule of furocoumarin. [b] $1/n$ defines, according to Mc Ghee and von Hippel,[18] the frequency of binding sites; in other words, it is the number of ligands bound per nucleotide and is analogous to the "n" value obtained by the classic Scatchard method. [c] Values concerning 1 from ref 10. [d] Values concerning 8-MOP from ref 19.

6-acetyl derivatives was carried out; fractional crystallization of the rearrangement products is not sufficient to eliminate completely the 6-isomers from the 8-acetyl derivatives, as shown by HPLC. During the successive synthetic steps, linear furocoumarins (psoralens) can be obtained from the 6-acetylumbelliferones.[12,13] This should be absolutely avoided because psoralens are able to photoinduce in DNA interstrand cross-linkages,[14] in other words, photolesions that our planned compounds should not be able to do.

The pure 8-acetylumbelliferones were treated with ethyl bromoacetate, and the resulting (7-coumarinyloxy)acetates (**6a–c**) were hydrolyzed to give the corresponding free acids (**7a–c**). By cyclization of **7a–c**, accompanied by an almost complete decarboxylation, the desired methylangelicins were obtained.

Noncovalent Binding to DNA. In a way similar to the other methylangelicins previously studied,[10,15] **2a–c** form a complex in the ground state with DNA, as shown by their aqueous solutions that, when examined in the presence of the macromolecule, show a marked decrease of the UV absorption capacity, a red shift of the λ_{max} of the coumarinic band around 300 nm, and a marked quenching of the fluorescence.

Taking into account that this event is a preliminary, necessary step for the successive photoaddition to DNA,[10,15,16] we have evaluated the binding parameters of these complexes. We have, therefore, followed the binding process of **2a–c** with DNA by equilibrium dialysis experiments by using tritiated angelicins as was done with previous angelicins.[10]

From the binding data, the values of r (molecules of ligand bound per nucleotide) and c (ligand free in the system, moles/liter) have been calculated according to Peacocke and Skerrett.[17]

The Scatchard plots of the dark binding of **2a–c** to DNA are reported in Figure 1. The binding isotherms reported in Figure 1, as well as the binding parameters K (association constant to an isolated site), n (the number of nucleotides occluded by a bound angelicin), and $1/n$ (the frequency of the binding site, i.e., the number of molecules of angelicin bound to every nucleotide) reported in Table I, have been calculated according to the method of Mc Ghee and von Hippel[18] on the basis of the experimental data of r and c.

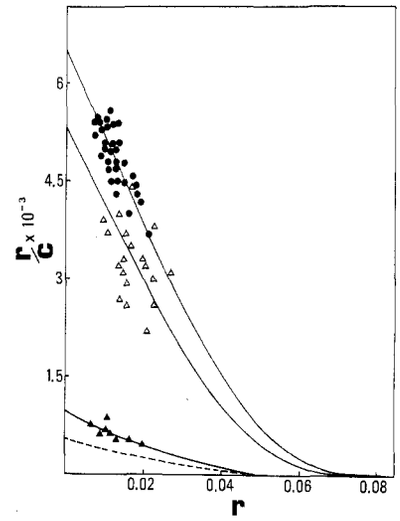

Figure 1. Scatchard plots for the binding in the dark of **2a** (▲) **2b** (●), **2c** (△), and 1 (dotted line, from ref 10) as reference compound, to calf thymus DNA. The curves were calculaded by a computer according to the method of Mc Ghee and von Hippel on the basis of the experimental values of r and c reported in the figure.

These data indicate that introduction of one methyl group in the 4'-position leads to an evident increase of the affinity toward DNA in comparison with the parent compound. On the other hand, introduction of a second methyl group in addition to that in 4'-position leads to a further increase of the binding to DNA, much higher than that observed in the previous angelicins and similar only to that of 4',5,8-trimetylpsoralen.[19]

Taking into account the analogy of the complexes between **2a–c** and DNA and those of the previous angelicins,[10,15] as well as those of psoralens,[20] it is reasonable to assume that these compounds, when complexed with DNA, would be intercalated between two base pairs of the macromoleule.

Water Solubility and Partition Coefficient. It has been previously observed that introduction of one or two methyl groups into the molecule of angelicin provoked a

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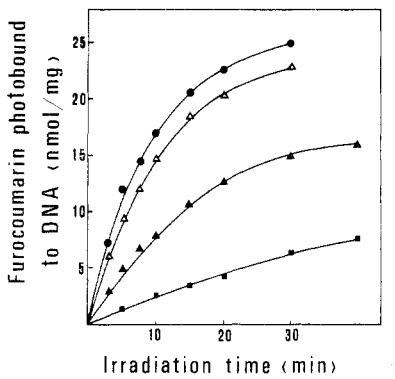

Figure 2. Photobinding of **2a** (▲), **2b** (●), **2c** (△), and **1** (■), as reference compound, toward calf thymus DNA.

decrease of the water solubility.[10] Taking into account this property as an indication of the variation of the hydrophobicity of the compounds in comparison with the parent angelicin (1), a somewhat inverse correlation between water solubility and the capacity to form the complex with DNA was observed.[10] However, to have a more precise indication of the possible variation of the hydrophobicity of 1 as a consequence of the introduction of the methyl groups, we have evaluated the octanol/water partition coefficients of 1 and the new compounds (see Table I). It can be seen in Table I that a good correlation exists between the partition coefficients and the corresponding affinities toward DNA in terms of association constants of the complexes.

The variation of hydrophobicity seems to be a factor that affects the affinity of these compounds toward the internal lipophilic part of the duplex DNA where intercalation occurs. Other factors, however, such as the electronic and steric effects connected with the introduction of the methyl groups in 1 can also play some role in this connection.

Photobinding to DNA. It is known that, in practice, only the angelicins intercalated in duplex DNA can photobind to the macromolecule,[9-11,15,21] for geometrical reasons, however, these compounds can engage only one of their two photoreactive sites (the 3,4, or 4',5' double bond) in the photocycloaddition with the pyrimidine bases of the DNA,[14] behaving, therefore, as monofunctional photoreagents toward the macromolecule.[9-11,15,21]

We have confirmed the monofunctional photobinding of **2a–c** with DNA experimentally. In fact, the DNA samples irradiated (1 h) in the presence of these three angelicins (10 μg/mL) did not show any renaturation capacity after heat denaturation, showing that no interstrand cross-linkages have been formed in the photoreactions. The formation of interstrand cross-linkages in DNA induces a marked renaturation capacity to the macromolecule.[14] The photobinding of **2a–c** to DNA is reported in Figure 2. These photoreactions, analogous to those between previous methylangelicins and DNA,[10] behave as pseudo-first-order reactions with respect to the complexed furocoumarin. The rate constant values are reported in Table II.

The introduction of one methyl group in the 4'-position leads to a marked increase of the photobinding to DNA in comparison with the parent compound **1**. Taking into account previous photobinding data obtained with various methylangelicins[10] (see Table II), we can see that the position where the methyl group is introduced affects the

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Table II. Rate Constants of the Photoreactions between Various Furocoumarins and DNA

compd	rate constant, min^{-1}
angelicin (1)	1.1×10^{-2}
4'-methylangelicin (2a)	3.7×10^{-2}
4-methylangelicin[a]	1.6×10^{-2}
5-methylangelicin[a]	3.4×10^{-2}
5'-methylangelicin[a]	2.1×10^{-2}
4',4-dimethylangelicin (2b)	7.3×10^{-2}
4',5-dimethylangelicin (2c)	5.9×10^{-2}
8-MOP	3.1×10^{-2}

[a] From ref 10.

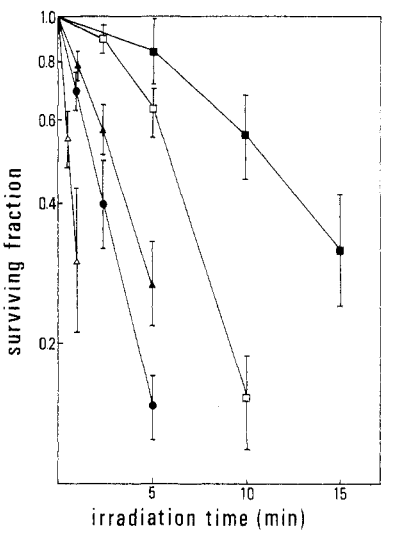

Figure 3. Inactivation of T_2 phage by irradiation (365 nm) in the presence of **2a** (▲), **2b** (●), **2c** (△), **1** (■), and 8-methoxypsoralen (8-MOP) (□). After irradiation, the plaque forming per milliliter were scored by using *E. coli* B_{48} as indicator bacteria. Incubation in the dark in the presence of the drugs and 40-min irradiation in their absence were both unable to reduce significantly the surviving fraction.

increase of the photobinding to DNA strongly; the role of the various positions, in fact, has the following order of importance: 4' > 5 > 5' > 4.

The introduction of a second methyl group leads to a further strong increase of the photobinding to DNA. This increase, however, does not correspond to the simple addition of the effects of the two methyl groups evaluated separately: the 4',4-dimethyl derivative (**2b**), in fact, photobinds to DNA more effectively than 4',5-dimethylangelicin (**2c**). These findings are in line with previous observations made with other dimethylangelicins.[10]

Inhibition of T_2 Phages. The photobiological activity of the 4'-methyl derivatives was tested on the basis of their capacity to photoinactivate the T_2 phage. A suspension of the phage was irradiated in the presence of **2a–c**, and for reference compounds, also in the presence of 1 and 8-methoxypsoralen (8-MOP); after irradiation, the infectivity of the phage on *E. coli*, as guest bacteria, was tested. The surviving curves of the phage as a function of time of irradiation are reported in Figure 3.

We can see that all three new compounds carrying a methyl group in the 4'-position show a strong increase of the activity in comparison with the unsubstituted **1**. In particular for derivatives **2b** and **2c**, where a second methyl group is present in the 4- and the 5-position, respectively, a further increase of the T_2 phage inhibition capacity is observed. The three new compounds show an activity even higher than that of 8-MOP.

Table III. Inhibition of Epidermal DNA Synthesis in the Mouse "in Vivo" by Topical Application or by Oral Administration and Irradiation at 365 nm[a]

	topical		oral	
furocoumarin	% inhibn ± SE	rel act. (8-MOP)	% inhibn ± SE	rel act. (8-MOP)
no drug, solvent alone	2.50 ± 1.3		3.20 ± 1.8	
1	17.50 ± 2.50	0.28	28.00 ± 2.0	0.71
2a	58.43 ± 7.91	0.95	36.75 ± 0.99	0.94
2b	44.27 ± 8.2	0.72	25.82 ± 8.38	0.66
2c	58.41 ± 2.41	0.95	53.33 ± 10.50	1.36
8-MOP	61.00 ± 1.6	1	39.10 ± 3.7	1

[a] Reference 22.

Inhibition of Epidermal DNA Synthesis in Mice. To have a further indication of the antiproliferative activity of the new compounds, we have evaluated their capacity to inhibit the synthesis of epidermal DNA on mouse skin after topical application or oral administration (see Table III) and UV-A irradiation, according to Bordin et al.[22]

We can see that by topical application and UV-A irradiation, the new 4'-methylangelicins **2a–c** show a marked capacity to inhibit epidermal DNA synthesis to an extent much higher than that of the parent 1. While **2b** shows an activity lower than 8-MOP, **2a** and **2c** inhibit epidermal DNA synthesis in a way strictly similar to that of this linear furocoumarin.

The data concerning the inhibition of epidermal DNA synthesis in mice after oral administration of the compounds and successive UV-A irradiation are reported in Table III. While **2a** and **2c** show inhibition capacities markedly higher than the parent **1**, **2b** shows an activity even a little lower than parent 1. This behavior may be explained in terms of pharmacokinetics, taking into account that different rates of absorption and/or excretion can occur with different furocoumarins.[23] In comparison with 8-MOP, **2a** shows an activity similar, even slightly lower, than this linear furocoumarin, while **2c** exhibits an activity markedly higher.

Genotoxicity. One of the side effects of photochemotherapy with psoralens is the risk of skin cancer.[5] Taking into account that for some furocoumarins, such as 8-MOP and 3-carbethoxypsoralen (3-CPs), the photomutagenic activity correlates with the capacity to photoinduce skin cancer in mice,[24] we have evaluated the mutagenicity of **2a–c** on *E. coli* WP2 strain under UV-A irradiation.

The mutants induced on *E. coli* WP2 by **2a–c** and, for a direct comparison, by 8-MOP, as a function of the time of irradiation with UV-A light, are reported in Figure 4. We can see that the mutagenic activity of the 4'-methyl derivatives is markedly lower than that of 8-MOP. However, among the three angelicins, **2a** appears more mutagenic than **2b** or **2c**. In this connection, no correlation between the capacity to induce photolesions in DNA and mutagenic activity can be observed.

On the other hand, these compounds tested in the dark on *Salmonella typhimurium* TA 98 did not show any mutagenic activity even after metabolic activation.[25] In other words, mutagenic activity of these compounds can be evidenced only after UV-light activation.

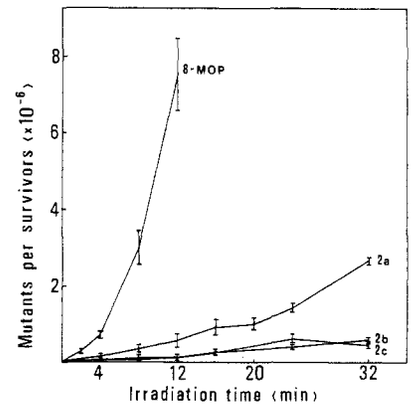

Figure 4. Mutagenic action of **2a–c** and 8-methoxypsoralen, as reference compound, on *E. coli* WP_2.

Clinical Data. The therapeutic effectiveness of **2c** that we have chosen among the three 4'-methyl derivatives on the basis of its antiproliferative activity, relatively low genotoxicity, and absence of skin phototoxicity on guinea pig skin[26] was tested by measuring its capacity to resolve psoriasis in two patients.

For a comparative evaluation, the efficacy of 8-MOP (8-methoxypsoralen), the agent most used for photochemotherapy, has also been tested under the same experimental conditions.

In the two patients treated, various areas 4 × 4 cm of the affected skin were used: (a) in the first area, an ethanolic solution (0.1% w/v) of **2c** was applied until a concentration of 20 $\mu g/cm^2$ was reached, and it was left to evaporate by the heat of the body (or by hot air stream). After 45 min, the area was irradiated with a high-intensity, UV-A emitting, low-pressure mercury fluorescent lamp, type PUVA, Waldmann Sylvania F 15 T 8. The irradiation doses were selected in the range between 2.5 and 13 J/cm^2; in particular, the initial dose was 2.5 J/cm^2, and this was gradually increased until it reached 13 J/cm^2. (b) In a second area, an ethanolic solution of 8-methoxypsoralen (8-MOP) was applied to a concentration of 20 $\mu g/cm^2$, and the area was irradiated in a strictly similar way as for **2c**. (c) a third area was treated in the same way as area a but was not irradiated with UV-A. (d) a fourth area was irradiated as in area a with the same dose of UV-A light in the absence of any compound. The treatment with 4',5-dimethylangelicin (**2c**) was repeated 5 times a week for 3 weeks. A good clearing of the psoriasis was observed after six treatments. In the case of 8-MOP, clearing was observed after ten treatments. While the area treated with the angelicin derivative but not with UV-A did not show any improvement, the area treated with UV-A alone showed a very low improvement. Compound **2c** induced

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a marked dark pigmentation in the treated area, more pronounced than that induced by 8-MOP under the same conditions, but showed practically no skin phototoxicity. In contrast, 8-MOP in one of the two patients treated, who showed a skin more sensitive to UV light (skin type II[27]), induced a marked erythema at the irradiation dose of 10 J/cm^2.

Conclusions

We have prepared three 4'-methylangelicins taking into account the necessity for the absence of their corresponding linear isomers (psoralens); these compounds can be originated from the 8-acetylumbelliferones, present as impurities in the key intermediates 6-acetylumbelliferones, during the successive synthetic steps. In this connection, it is well-known that the presence of even small amounts of the linear bifunctional methylpsoralens can modify markedly the photobiological properties of the corresponding methylangelicins.[28]

The increased affinity of **2a–c** in comparison with the parent compound **1** toward DNA for the dark complex formation appears well correlated with their increase of hydrophobicity, as shown by the partition coefficients in the octanol/water system. A variation of hydrophobicity seems to be a factor that can affect the extent of complexation of these compounds with the macromolecule.

Compounds **2a–c** in the presence of DNA and activated by UV-A photobind monofunctionally to the macromolecule much more effectively than angelicin (**1**) and even more than 8-MOP. These compounds effectively block the infectivity of T_2 phages and are able to inhibit epidermal DNA synthesis in mice by both oral administration and topical application. Moreover, these compounds practically do not show skin phototoxicity at the therapeutical concentrations; only **2a** and **2b** at high concentrations and with high doses of UV light have been able to induce erythema on guinea pig skin.[26,29]

Compounds **2a–c** show a genotoxicity much lower than that of 8-MOP. A preliminary clinical evaluation of **2c**, realized on two patients, showed that this compound appears effective in clearing psoriasis. The low genotoxicity, the absence of skin phototoxicity, and the preliminary therapeutic effectiveness of **2c** suggest this compound as a potential drug for the topical photochemotherapy of psoriasis. Further clinical and toxicological studies are, however, required.

Experimental Section

Melting points were determined in open capillary tubes and are uncorrected. TLC was performed on precoated Merck 60-F-254 silica gel plates (0.25 mm) and developed with ethyl acetate/cyclohexane (35:65). Preparative column chromatography was performed with silica gel (Merck; 0.063–0.200 mm). HPLC was performed on a Perkin-Elmer Series 3 apparatus, equipped with a LC 75 detector at 250 nm, by using a silica A column (Perkin-Elmer; 0.26 × 25) eluting under isocratic conditions with hexane/$CHCl_3$/MeOH (76:19:5; 2 mL/min) (system A) or by using a Lichrosorb RP-18 column (Merck; 10 μm; 0.4 × 25) eluting under isocratic conditions with MeOH/H_2O (6:4; 2 mL/min) (system B). ^{1}H NMR spectra were recorded on a Varian FT-80 A spectrometer with Me_4Si as internal standard (δ 0) and $CDCl_3$ as solvent, unless otherwise indicated; coupling constants are given in hertz. The relative peak areas and decoupling experiments were in agreement with all the assignments. All products give satisfactory elemental analyses (within ±0.3%). UV spectra were recorded on a Perkin-Elmer Model 554 spectrometer.

Fries Rearrangement. An accurately mixed mixture of 7-acetoxycoumarin (**3a**; 5.0 g, 24.5 mmol), anhydrous aluminum chloride (10.0 g, 74.9 mmol), and anhydrous sodium chloride (2.0 g, 34.4 mmol) was heated at 170 °C for 1.5 h. After the addition of dilute HCl (50 mL), the cooled mixture was refluxed for 10 min, diluted with water (200 mL), and extracted three times (100 mL) with ethyl acetate.

The solvent was evaporated from the dried (Na_2SO_4) organic phase, and the solid residue was chromatographed on a silica gel column by eluting with $CHCl_3$. In the fractions initially eluted, the solvent was evaporated, yielding the 7-hydroxy-8-acetylcoumarin (**4a**) free from the 6-isomer (**5a**) (HPLC, system A): total yield 3.2 g (64%); mp 179–180 °C (from MeOH); ^{1}H NMR δ 13.62 (1 H, s, which disappeared by D_2O addition, 7-OH), 7.65 (1 H, d, J = 9.5, 4-H), 7.53 (1 H, d, J = 8.8 Hz, 5-H), 6.90 (1 H, d, J = 8.8 Hz, 6-H), 6.27 (1 H, d, J = 9.5 Hz, 3-H), 2.96 (3 H, s, $COCH_3$).

When the elution was continued, a number of fractions containing both isomers were collected, followed by a number of fractions from which pure 6-acetyl-7-hydroxycoumarin (**5a**) was isolated: yield 0.25 g (5%); mp 176–177 °C (from MeOH); ^{1}H NMR δ 12.65 (1 H, s, which disappeared by D_2O addition, 7-OH), 7.89 (1 H, s, 5-H), 7.63 (1 H, d, J = 9.6 Hz, 4-H), 6.85 (1 H, s, 8-H), 6.29 (1 H, d, J = 9.6 Hz, 3-H), 2.68 (3 H, s, $COCH_3$).

In a similar manner, from 4-methyl-7-acetoxycoumarin (**3b**; 5.0 g, 22.9 mmol) was obtained 4-methyl-7-hydroxy-8-acetylcoumarin (**4b**), free (HPLC, system A) from the 6-isomer (**5b**): yield 3.1 g (62%); mp 166 °C (from MeOH); ^{1}H NMR δ 13.56 (1 H, s, which disappeared by D_2O, 7-OH), 7.67 (1 H, d, J = 8.9 Hz, 5-H), 6.92 (1 H, d, J = 8.9 Hz, 6-H), 6.17 (1 H, q, J = 1.0 Hz, 3-H), 2.96 (3 H, s, $COCH_3$), 2.42 (3 H, d, J = 1.0 Hz, 4-CH_3).

4-Methyl-6-acetyl-7-hydroxycoumarin (**5b**) was also isolated: yield 0.152 g (3%); mp 205 °C (from MeOH); ^{1}H NMR δ 12.61 (1 H, s, which disappeared by D_2O addition, 7-OH), 7.96 (1 H, s, 5-H), 6.84 (1 H, s, 8-H), 6.17 (1 H, q, J = 1.2 Hz, 3-H), 2.70 (3 H, s, $COCH_3$), 2.44 (3 H, d, J = 1.2 Hz, 4-CH_3).

From 5-methyl-7-acetoxycoumarin (**3c**; 5.0 g, 22.9 mmol) was obtained 5-methyl-7-hydroxy-8-acetylcoumarin (**4c**), free (HPLC, system A) from the 6-isomer (5c): yield 2.35 g (47%); mp 189–190 °C (from MeOH) (lit.[12] mp 190–191 °C); ^{1}H NMR δ 13.66 (1 H, s, which disappeared by D_2O addition, 7-OH), 7.83 (1 H, d, J = 9.8, 4-H), 6.73 (1 H, s, 6-H), 6.27 (1 H, d, J = 9.8 Hz, 3-H), 2.92 (3 H, s, $COCH_3$), 2.48 (3 H, s, 5-CH_3).

In this case the crude rearrangement product showed (TLC) that very low amounts of the isomeric 6-acetyl-derivative were present, which were not isolated in pure form; on the contrary a larger amount of pyrolized product was present, that is the starting 5-methylumbelliferone.

Ethyl (7-Coumarinyloxy)acetates. An acetonic solution (150 mL) of 7-hydroxy-8-acetylcoumarin (**4a**; 3.0 g, 14.7 mmol), ethyl bromoacetate (3 mL, 27 mmol), and anhydrous K_2CO_3 (4.0 g) was refluxed for 4 h. The solid was filtered from the chilled mixture and washed with acetone, and the solvent was evaporated. The residue was crystallized from MeOH to give ethyl [(8-acetyl-7-coumarinyl)oxy]acetate (**6a**): yield 3.0 g (70%); mp 123–124 °C; ^{1}H NMR δ 7.64 (1 H, d, J = 9.6 Hz, 4-H), 7.14 (1 H, d, J = 8.6 Hz, 5-H), 6.77 (1 H, d, J = 8.6 Hz, 6-H), 6.26 (1 H, d, J = 9.6 Hz, 3-H), 4.74 (2 H, s, OCH_2), 4.25 (2 H, q, J = 7.1, $COOCH_2$), 2.64 (3 H, s, $COCH_3$), 1.28 (3 H, t, J = 7.1 Hz, $COOCH_2CH_3$).

In a similar manner, from 4-methyl-7-hydroxy-8-acetylcoumarin (**4b**; 1.8 g, 8.2 mmol) was obtained ethyl [(4-methyl-8-acetyl-7-coumarinyl)oxy]acetate (**6b** 1.45 g (58%); mp 116 °C (from MeOH); ^{1}H NMR δ 7.57 (1 H, d, J = 9.0 Hz, 5-H), 6.78 (1 H, d, J = 9.0 Hz, 6-H), 6.14 (1 H, q, J = 1.1 Hz, 3-H), 4.75 (2 H, s, $OOCH_2$), 4.25 (2 H, q, J = 7.1 Hz, OCH_2CH_3), 2.63 (3 H, s, $COCH_3$), 2.39 (3 H, d, J = 1.1 Hz, 4-CH_3), 1.29 (3 H, t, J = 7.1 Hz, OCH_2CH_3).

From 5-methyl-7-hydroxy-8-acetylcoumarin (**4c**; 1.0 g, 4.6 mmol) was obtained ethyl [(5-methyl-8-acetyl-7-coumarinyl)-oxy]acetate (**6c**): yield 1.1 g (78%); mp 131–132 °C (from MeOH) (lit.[12] mp 130–132 °C); ^{1}H NMR δ 7.81 (1 H, d, J = 9.8, 4-H), 6.58 (1 H, s, 6-H), 6.25 (1 H, d, J = 9.8 Hz, 3-H), 4.72 (2 H, s, $OOCH_2$), 4.27 (2 H, q, J = 7.2 Hz, OCH_2CH_3), 2.62 (3 H, s, $COCH_3$), 2.49

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(3 H, s, 5-CH_3), 1.29 (3 H, t, J = 7.2 OCH_2CH_3).

(7-Coumarinyloxy)acetic Acid. An aqueous 5% KOH methanolic (1:1, 100 mL) solution of ethyl [(8-acetyl-7-coumarinyl)oxy]acetate (**6a**; 4.0 g, 13.5 mmol) was refluxed for 15 min in the dark. After chilling, the mixture was acidified with dilute HCl, water (200 mL) was added, and the solid was filtered, washing it many times with water. The solid was crystallized from ethyl acetate and identified as [(8-acetyl coumarinyl)oxy]acetic acid (**7a**): yield 2.1 g (59%); mp 218–219 °C; ^{1}H NMR [$(CD_3)_2CO$] δ 14.06 (1 H, br s, COOH), 8.03 (1 H, d, J = 9.6 Hz, 4-H), 7.79 (1 H, d, J = 8.8 Hz, 5-H), 7.19 (1 H, d, J = 8.8 Hz, 6-H), 6.39 (1 H, d, J = 9.6 Hz, 3-H), 5.07 (2 H, s, OCH_2COOH), 2.60 (3 H, s, $COCH_3$).

In the same manner, from ethyl [(4-methyl-8-acetyl-7-coumarinyl)oxy]acetate (**6b**; 1.0 g, 3.3 mmol) was obtained [(4-methyl-8-acetyl-7-coumarinyl)oxy]acetic acid (**7b**): yield 0.64 g (70%); mp 218 °C (from MeOH); ^{1}H NMR [$(CD_3)_2CO$] δ 13.60 (1 H, br s, COOH), 7.88 (1 H, d, J = 9.0 Hz, 5-H), 7.20 (1 H, d, J = 9.0 Hz, 6-H), 6.29 (1 H, q, J = 1.2 Hz, 3-H), 5.06 (2 H, s, OCH_2COOH), 2.69 (3 H, s, $COCH_3$) δ 2.56 (3 H, d, J = 1.2 Hz, 4-CH_3).

From ethyl [(5-methyl-8-acetyl-7-coumarinyl)oxy]acetate (**6c**; 0.9 g, 2.9 mmol) was obtained [(5-methyl-8-acetyl-7-coumarinyl)oxy]acetic acid (**7c**): yield 0.6 g (75%); mp 229 °C (from MeOH) (lit.[12] 225–227 °C); ^{1}H NMR [$(CD_3)_2CO$] δ 8.20 (1 H, d, J = 9.9 Hz, 4-H), 7.10 (1 H, br s, 6-H), 6.37 (1 H, d, J = 9.9 Hz, 3-H), 5.04 (2 H, s, OCH_2COOH), 2.68 (3 H, s, $COCH_3$), 2.67 (3 H, br s, 5-CH_3).

Cyclization. A mixture of [(8-acetyl-7-coumarinyl)oxy]acetic acid (**7a**; 2.0 g, 7.6 mmol), Ac_2O (30 mL), and anhydrous sodium acetate (5.0 g) was refluxed for 1 h; to the chilled mixture was cautiously added water (30 mL), and the mixture was refluxed for 10 min, diluted with water (200 mL), and extracted with ethyl acetate. The organic phase was evaporated under vacuum, the solid was redissolved in EtOAc, and the organic phase was washed three times with saturated $NaHCO_3$ solution (50 mL).

The solvent was evaporated from the dried organic phase, and the residue was crystallized from MeOH to give the pure (HPLC, system B) 4'-methylangelicin (**2a**): yield 0.78 g (51%); mp 150 °C; ^{1}H NMR δ 7.78 (1 H, d, J = 9.6 Hz, 4-H), 7.42 (1 H, q, J = 1.3 Hz, 5'-H), 7.33 (2 H, s, 5-H and 6-H), 6.35 (1 H, d, J = 9.6 Hz, 3-H), 2.52 (3 H, d, J = 1.3 Hz, 4'-CH_3).

The pooled alkaline washings were acidified with dilute HCl and extracted many times with ethyl acetate. The dried (Na_2SO_4) organic phase was shown by TLC to contain a single spot that did not move in the developed plate. The poor residue obtained by evaporation of the solvent (0.035 g, 2%) had not melted up to 310 °C and was considered to be the undecarboxylated intermediate product **8a**.

In a similar manner, from [(4-methyl-8-acetyl-7-coumarinyl)-oxy]acetic acid (**7b**; 1.7 g, 6.1 mmol) was obtained pure [HPLC, silica A column (Perkin-Elmer, 0.26 × 25), eluent $CHCl_3$ (2 mL/min)] 4',4-dimethylangelicin (**2b**): yield 0.9 g (69%); mp 178–179 °C (from MeOH) (lit.[13] mp 154 °C); ^{1}H NMR δ 7.42 (1 H, d, J = 8.7 Hz, 5-H), 7.39 (1 H, q, J = 1.4 Hz, 5'-H), 7.32 (1 H, d, J = 8.7 Hz, 6-H), 6.20 (1 H, q, J = 1.2 Hz, 3-H), 2.49 (3 H, d, J = 1.4 Hz, 4'-CH_3), 2.45 (3 H, d, J = 1.2 Hz, 4-CH_3).

from [(5-methyl-8-acetyl-7-coumarinyl)oxy]acetic acid (**7c**; 0.5 g, 1.8 mmol) was obtained pure (HPLC, system B) 4',5-dimethylangelicyn (**2c**): yield 0.3 g (78%); mp 173–174 °C (from MeOH) (lit.[12] 169–170 °C); ^{1}H NMR δ 7.93 (1 H, d, J = 9.8 Hz, 4-H), 7.31 (1 H, q, J = 1.3 Hz, 5'-H), 7.13 (1 H, q, J = 0.8, 6-H), 6.32 (1 H, d, J = 9.8 Hz, 3-H), 2.55 (3 H, d, J = 0.8 Hz, 5-CH_3), 2.46 (3 H, d, J = 1.3, 4'-CH_3).

DNA. Calf thymus DNA (cat. D 1501) was purchased from Sigma Chemical Co., St. Louis; hypochromicity of the sample, determined according to Marmur and Doty,[30] was higher than 40%.

Equilibrium Dialysis Experiments. Cylindrical containers, 4-cm diameter, 1.6-cm depth, divided into two parts by a cellophane membrane (Visking Corp., U.S.A.) have been used; in one part of the cell, the aqueous solution of a labeled furocoumarin containing NaCl (0.02 mol) and EDTA (1 mmol) at a concentration a little under its water solubility was introduced; in the other part of the cell, aqueous DNA solutions in the presence of the same labeled angelicin, at the same ionic strength, having decreasing concentrations in the range between 3×10^{-3} and 3×10^{-4} mol, were introduced. In these DNA solutions the initial concentration of the angelicin was constant and identical with that of the aqueous phase.

The cells were mechanically shaken for 12 h in a thermostat at 25 ± 0.05 °C in the dark.[10]

After the shaking period, small volumes (0.2 mL) of the two phases were utilized for radiochemical measurements: in this way, the concentrations of the furocoumarin in the two phases were determined, and r and c values were calculated.[17]

In order to evaluate the extent of furocoumarin complexed to DNA (100% of reactant at 0 times) for calculating the rate constants of the photoreactions,[10] analogous experiments have been made, but at lower ionic strength (2 mmol NaCl); moreover, the concentrations of the furocoumarins and DNA were varied so that in the DNA phase, once equilibrium was reached, the ratio of ligand to nucleotides (1:76) was strictly similar to that of the solutions used for the photobinding experiments.

Radiochemical Determinations. A liquid scintillation spectrometer (Packard Model A 300 CD) was used. Small volumes (0.2 mL) of the solutions in which the concentration of furocoumarin had to be determined were added to 10 mL of dioxane base scintillator (PPO, 5 g; POPOP, 0.075 g; naphthalene, 120 g; dioxane, up to 1000 mL of solution) and then counted. The apparatus efficiency for counting tritium was within the range 35–65%. Compounds **2a–c** have been labeled according to ref 10; they had the following specific radioactivity: 8.9, 11.5, and 8.3 Ci/mol, respectively.

Computation of the Interaction Parameters. The method of computation involved an iterative procedure able to satisfy the following equation of Mc Ghee and von Hippel:[18]

$$\frac{r}{c} = K(1 - nr)\left(\frac{1 - nr}{[1 - (n - 1)]r}\right)^{n-1}$$

given the experimentally determined values of r and c and the initial guess of K (the intrinsic binding constant to an isolated site) and of n (the number of nucleotides occluded by a bound furocoumarin molecule). The program, based on the least-squares method of Taylor series expansion of the above-reported equation, was made to recycle until K and n changed by less than 1% and then, to give the final values of K and n, with a calculated binding isotherm at 5% saturation increments.

Determination of Partition Coefficient. A solution (5 mL) of the compound (30 μg/mL) in octanol was shaken with 5 mL of water in a 50-mL separatory funnel for 4 h. The layers were separated, the concentration was determined by radiochemical measurements, and the partition coefficient was calculated as $S_{octanol}/S_{water}$.

Irradiation Procedure. Aqueous solutions (2.3×10^{-3} mol) of DNA containing 2×10^{-3} mol of NaCl and 1×10^{-3} mol of EDTA were added to the labeled furocoumarin to be examined (3×10^{-5} mol). Measured volumes (2 mL) of the prepared solutions were introduced into calibrated glass tubes, and these were immersed into a thermostatically controlled bath and irradiated for constantly increasing periods of time by means of two HPW 125 Philips lamps (which emit almost exclusively at 365 nm[31]); the irradiation intensity, determined by using a chemical actinometer,[32] was 1.07×10^{16} quanta s^{-1} mL^{-1}. After irradiation, the macromolecule was precipitated by addition of NaCl (2 mol) and absolute ethanol (2 vol), and the precipitate was washed with 80% ethanol and redissolved in the initial volume of water. The solutions so obtained were used for radiochemical determinations.[10]

Evaluation of Cross-linkages. This evaluation was made directly on the irradiated DNA samples, without precipitation, according to ref 10.

T_2 Phage Inactivation. T_2 phage was grown by using *E. coli* B_{48} as host bacteria and the brain heart infusion broth (from Difco

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Laboratoires, MI); virions were irradiated in Hanks solution at a density of 10^{10} particles per milliliter, as reported above for the Ehrlich cells, and in the presence of 4 μg/mL of the tested compounds. Virus titers were determined according to Adams,[33] using the same host and the same medium.

Inhibition of Epidermal DNA Synthesis in Mice. (a) By Topical Application. Furocoumarins were applied on the depilated skin of the backs of female mice by using 0.1% methanolic solutions (50 μg cm^{-2}); every compound was tested on at least six mice. The animals were then kept in a dark room for 45 min; the backs were then irradiated with UV-A light (9 J cm^{-2}, delivered in 30 min). Immediately after irradiations, [^{3}H]thymidine (10 μCi) was injected intraperitoneously; after 30 min the mice were sacrified.

The skin specimens were removed, and the epidermis was isolated according to the method of Bowden et al.,[34] with slight modifications; the whole skin was kept for 1 min in distilled water at 55 °C and chilled by immersion in ice-cold water. The skin was placed on a cooled (-20 °C) glass plate, dermis side down, and the epidermis was removed by scraping with a razor blade. The epidermis was cut with a scissor, suspended in 10 vol of ice-cold sodium saline citrate (0.15 mol of sodium chloride; 0.02 mol of sodium citrate), and then homogenized with a Vortex mixer and a Potter-Elvehjn homogenizer, with cooling in ice. The mixture was made 2% in sodium lauryl sulfate and 1 mol in sodium chloride and then left at room temperature for 2 h; the suspension was extracted three times with a chloroform–butanol (4:1) mixture (v/v), according to the method of Szybalska and Szybalski.[35]

The DNA was then precipitated with ethanol, washed with 80 °C ethanol, and dissolved in water; the solution was analyzed for DNA content and radioactivity. The percent inhibition of DNA synthesis observed in each sample was calculated assuming as a control the radioactivity incorporation obtained in the epidermal DNA of the abdomen skin of the same animal, processed in the same way. The specific activities observed in the control skins were in the range of 0.9–1.2 × 10^4 dpm mg^{-1} of DNA.

(b) By Oral Administration. The mice (NCL, 20 ± 2 g in weight) were starved for 3 h before being given, by oral intubation, a single dose of 5 mg per mouse of the furocoumarin, suspended in 0.5% methylcellulose; in these experiments also, groups of at least six animals were used. The mice were kept in a dark room for 2 h and then the skin of the back was irradiated with UV-A (9 J cm^{-2}). The subsequent steps for epidermal cell isolation, homogenization, and DNA extraction were similar to those described above. The unexposed skin of the abdomen, protected from light by black paper, was assumed, as above, as a control.

Mutagenesis Test. Furocoumarins, dissolved in absolute ethanol, were stored in the dark at room temperature prior to use. After the addition of furocoumarins (4 μg/mL), the *Escherichia coli* WP2 *trp*$^-$ suspension (10^7 cells/mL) was stored in the dark for 15 min at room temperature and then irradiated with a blacklight blue fluorescent lamp (F15T8 BLB, 15 W). Irradiated cells (0.1 mL) were added to 2 mL of molten 0.6% top agar kept at 42 °C. The contents were mixed and poured on plates containing 20 mL of SEM agar (MMA fortified with 0.1 mg/mL of Difco nutrient broth).[36] Revertant colonies were counted after 48 h of incubation at 37 °C in the dark. Survivors were counted by plating appropriate dilutions of irradiated cells on the same medium.

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Registry No. **2a**, 78982-40-8; **2b**, 22975-76-4; **2c**, 5762-92-5; **3a**, 10387-49-2; **3b**, 2747-05-9; **3c**, 80813-61-2; **4a**, 6748-68-1; **4b**, 2555-29-5; **4c**, 6109-07-5; **5a**, 6835-55-8; **5b**, 16555-98-9; **5c**, 80813-62-3; **6a**, 80813-63-4; **6b**, 31479-62-6; **6c**, 5762-90-3; **7a**, 80813-64-5; **7b**, 22975-75-3; **7c**, 5762-91-4; **8a**, 80813-66-7; **8b**, 20052-10-2; **8c**, 80813-65-6.

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Synthesis and Antitumor Evaluation of Selected 5,6-Disubstituted 1(2)*H*-Indazole-4,7-diones[1]

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A series of novel aziridinyl-substituted 1(2)*H*-indazole-4,7-diones and related 1(2)*H*-indazole-4,7-diones was synthesized and tested against Ehrlich ascites carcinoma growth in male CF_1 mice. Ten of the test compounds, including two aziridinyl-substituted 1(2)*H*-indazole-4,7-diones, were found to be significantly active (inhibition of tumor growth >80%) in the Ehrlich ascites carcinoma screen. Several structure–activity relationships were indicated for antitumor activity in this screen. An aziridinyl-substituted derivative, 5-aziridinyl-6-chloro-1*H*-indazole-4,7-dione (**8a**), also exhibited significant activity against the growth of P-388 lymphocytic leukemia cells in male BDF_1 mice (% T/C = 145; % T/C > 125 is considered significant).

Many naturally occurring and synthetic compounds that contain the *p*-benzoquinone moiety have been investigated for antitumor activity.[2-16] Of these compounds, several have proven to be potent antineoplastic agents. For example, the anthracycline antibiotics are some of the most

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