New Monofunctional Reagents for DNA as Possible Agents for the Photochemotherapy of Psoriasis: Derivatives of 4,5'-Dimethylangelicin

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With the aim of obtaining new agents for the photochemotherapy of psoriasis, we have prepared monofunctional reagents for DNA by starting from 4,5'-dimethylangelicin (2), an angular furocoumarin, and introducing in a 4'-(hydroxymethyl) (3), 4'-(methoxymethyl) (4), or 4'-(aminomethyl) group (5), in way analogous to what other authors have done previously on trioxsalen, a DNA bifunctional reagent. These new compounds form complexes with DNA in the ground state and by successive irradiation (UV-A) undergo monofunctional photoaddition to the macromolecule. Photobinding to DNA was highest for 3 and gradually lower for 4 and 5, respectively. These compounds do not form interstrand photocross-linkages in DNA and do not show any skin phototoxicity. Fluorimetric studies show that their 4',5' double bond is involved in the photoaddition to DNA. Their photobiological activity evaluated on Ehrlich ascites tumor cells and on T_2 phages was strictly connected with their photobinding to DNA. The effect of the introduction of hydroxymethyl and methoxymethyl groups in angular 2 is somewhat similar to that previously described for trioxsalen: the introduction of an aminomethyl group in 2 markedly increases the affinity in the dark for DNA but under UV-A irradiation strongly inhibits photobinding to the macromolecule. By contrast, in the analogous derivative of trioxsalen both the affinity for DNA in the dark and the photobinding to DNA increased.

Some linear furocoumarins, also called psoralens, are used in the photochemotherapy of psoriasis and other skin diseases, characterized by hyperproliferation of the skin; this treatment is also called PUVA (psoralens + ultraviolet A) therapy. A linear furocoumarin (8-methoxypsoralen is commonly used) is administered orally or topically and then the skin of the patient is irradiated with long-wavelength light (>320 nm).[1-3] Photobiological, as well as therapeutical, activity of these substances is connected with the highly specific photodamages that they produce in DNA under UV-A irradiation. These photodamages inhibit nucleic acid synthesis and cell division, and to this action is ascribed the therapeutical benefits of photochemotherapy.[1]

In this connection, it is well known that, while linear furocoumarins (psoralens) produce both monofunctional and bifunctional lesions,[5-7] angelicin (1, the parent angular furocoumarin) manifests its photobiological activity to produce only monofunctional lesions.[6,8] This compound, however, shows low photobinding capacity toward DNA and low photobiological activity.[8]

Considering that in PUVA therapy some undesired side effects are present, such as skin phototoxicity and risk of skin cancer[9] mainly connected with bifunctional lesions, we have recently prepared and studied a new angular furocoumarin, 4,5'-dimethylangelicin (2) with the aim of obtaining an effective monofunctional reagent for DNA useful for the photochemotherapy of psoriasis: its high monofunctional photoreactivity towards DNA, its marked capacity of inhibiting cell division, and its complete absence of skin phototoxicity strongly support the use of this compound in the photochemotherapy of psoriasis and other skin diseases.[10,11]

In continuing this research with the aim of modifying the lipophilic character of 4,5'-dimethylangelicin (2), we have prepared some of its new derivatives carrying hydroxymethyl, methoxymethyl, and aminomethyl groups in 4' position in a strictly analogous way as Isaacs et al. made on a linear furocoumarins, i.e., 4,5',8-trimethylpsoralen (trioxsalen).[12] The new compounds have been studied to evaluate their capacity to form an intercalated complex with native DNA and to ascertain their ability to photobind through monofunctional additions to the same macromolecule. Their lack of skin phototoxicity was confirmed on guinea pig skin, while their photobiological activity has been evidenced on other biological substrates.

Results

Chemistry. 4'-(Hydroxymethyl)-4,5'-dimethylangelicin (3), 4'-(methoxymethyl)-4,5'-dimethylangelicin (4), and the hydrochloride of 4'-(aminomethyl)-4,5'-dimethylangelicin (5) have been prepared from 2 according to the synthetic procedure of Isaacs et al.[12] The tritiated compounds 3-5 have been prepared by substituting, in an analogous way as for unlabeled compounds, hydroxymethyl, methoxymethyl, or aminomethyl groups on tritiated compound 2, prepared as described elsewhere.[11]

Water Solubility. The water solubility of compound 5, reported in Table I, is strongly increased in comparison to that of the parent compound 2, showing a parallel behavior with the corresponding cationic derivative of trioxsalen, previously prepared.[12] The introduction of the hydroxymethyl group leads to an increase of the water solubility higher than that of the methoxymethyl group, even if for these compounds the extent of increase, in comparison with 2, is not strong (see Table I).

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(9) J. H. Epstein, *N. Engl. J. Med.*, **300**, 852 (1979).
(10) F. Bordin, F. Baccichetti, and F. Carlassare, *Z. Naturforsch., C*, **33**, 296 (1978).
(11) F. Bordin, F. Carlassare, F. Baccichetti, A. Guiotto, P. Rodighiero, D. Vedaldi, and F. Dall'Acqua, *Photochem. Photobiol.*, **29**, 1063 (1979).
(12) S. T. Isaacs, Che-Kun J. Shen, J. E. Hearts, and H. Rapoport, *Biochemistry*, **16**, 1058 (1977).

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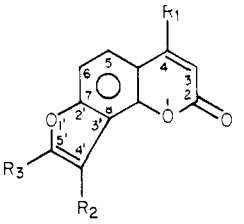
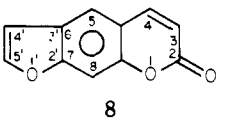
Table I. Angelicin Derivatives 1-7[a]



1-7



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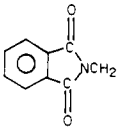
no.	R_1	R_2	R_3	formula	anal.	sp radioact.,[b] Ci/mol	water solubility[b] μg/mL	water solubility[b] mol/L
1	H	H	H			1.59	nd	nd
2	CH_3	H	CH_3			5.11	8.0	3.7×10^{-5}
3	CH_3	CH_2OH	CH_3	$C_{14}H_{12}O_4$	C, H	0.13	17.1	7.0×10^{-5}
4	CH_3	CH_2OCH_3	CH_3	$C_{15}H_{14}O_4$	C, H	0.21	15.3	5.6×10^{-5}
5	CH_3	$CH_2NH_2 \cdot HCl$	CH_3	$C_{14}H_{14}ClNO_3$	C, H, N, Cl	0.13	>1000.0	$>4.0 \times 10^{-3}$
6	CH_3	CH_2Cl	CH_3	$C_{14}H_{11}ClO_3$	C, H, Cl	nd	nd	nd
7	CH_3		CH_3	$C_{22}H_{15}NO_5$	C, H, N	nd	nd	nd

[a] The molecular structure of psoralen (8) is also reported for a comparison. [b] nd = not determined.

In the corresponding derivatives of trioxsalen, previously described,[12] while a parallelism exists on the relative effect of these two groups (the hydroxymethyl group, in fact, leads to a higher water solubility than the methoxymethyl group), a strong difference exists on the extent of the increase of the water solubility that is much more pronounced in the trioxsalen derivatives than in our compounds. This fact may be in part ascribed to the great difference of water solubility between our parent compound (2; 8.0 μg/mL) and that of trioxsalen (0.6 μg/mL[12]).

Molecular Complexes in the Ground State. It is well known that a preliminary complex in the ground state occurs between furocoumarins and duplex DNA and that this molecular complex plays an important role in the successive cycloaddition.[5-7,11-13]

Following the binding between the various angelicins and DNA, the molecular complexes have been studied by equilibrium dialysis experiments[13] or by fluorimetric titrations.[14] From the binding data the values of r (molecules of ligand bound per nucleotide) and c (ligand free in the system, mol/L) have been calculated according to Peacocke and Skerrett:[15] in Figure 1 these values are reported according to Scatchard, i.e., plotting r/c against r. The binding parameters of the complexes, i.e., K (association constant to an isolated site), n (number of nucleotides occluded by a bound angelicin), and $1/n$ (frequency of the binding sites; in other words, number of molecules of angelicin bound to every nucleotide), have been calculated according to the more recent method of Mc Ghee and Von Hippel.[16]

The introduction of a cationic group in the 4' position of compound 2, as for the hydrochloride of 4'-(aminomethyl)-4,5'-dimethylangelicin (5), strongly increases the binding parameters of the complex formed with DNA.

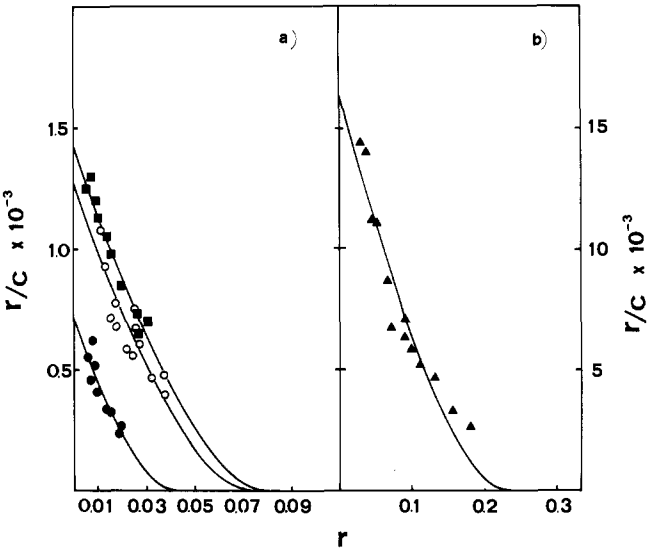


Figure 1. Scatchard plots for the binding in the ground state of 2 (■), 3 (○), 4 (●) (part a), and 5 (▲) (part b) to calf thymus DNA. The curves were calculated by a computer according to the method of McGhee and Von Hippel[16] on the basis of the experimental values of r and c reported in the figure.

Table II. Binding Parameters of the Complexes between the Angelicin Derivatives and Calf Thymus DNA

no.	K values	n[a]	$1/n$[b]
2	1400	10.6	0.095
3	1250	11.5	0.087
4	700	20.0	0.05
5	16 300	3.9	0.255

[a] According to Mc Ghee and Von Hippel;[16] n is defined here as the number of nucleotides occluded by one bound molecule of angelicin derivative. [b] $1/n$ defines, according to McGhee and Von Hippel, the frequency of binding sites; in other words, the number of ligands bound per nucleotide and can be considered analogous to the "n" value obtained by the classic Scatchard method.[34]

The association constant (K), in fact, is about 11 times higher than that of the parent, 2 (see Table II and Figure 1), and also the $1/n$ value is markedly increased. This

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(15) A. R. Peacocke and J. N. H. Skerrett, *Trans. Faraday Soc.*, 52, 261 (1956).
(16) J. D. Mc Ghee and P. H. Von Hippel, *J. Mol. Biol.*, 86, 469 (1974).

behavior is analogous to the corresponding cationic derivative of trioxsalen.[12] These data can be interpreted by considering that in the compounds carrying cationic groups two types of interaction should be involved in the binding with DNA: an external binding between the cationic head and phosphate groups of the macromolecule and an internal binding due to the intercalation of the planar tricyclic moiety between two base pairs of DNA. This double interaction, analogous to that of some antitumor antibiotics (e.g., daunomycin, adriamycin, actinomycin D, etc.) provokes the increased affinity of the two cationic compounds toward DNA in comparison to their parent, angular and linear furocoumarins.

When a hydroxymethyl group is introduced into the 4′ position (3), the affinity in the dark for DNA complex formation is only slightly lowered in comparison with the parent 2; on the other hand, when a methoxymethyl group is introduced into the same position (4), the extent of complexation is markedly decreased (See Table II and Figure 1). This behavior is also somewhat similar to that observed in the corresponding derivatives of trioxsalen, where the introduction of the same groups decreases the affinity in the dark for DNA complex formation as shown by the increased values of the dissociation constants of the corresponding complexes with the macromolecule.[12]

Photoaddition to DNA. A DNA aqueous solution was irradiated in the presence of the various angelicins for increasing periods of time. After irradiation, DNA was precipitated with ethanol and redissolved in the initial volume with water.[5,6,11] The solution obtained was divided into two parts: part A was utilized for radiochemical measurements for evaluating the photobinding to DNA, while part B was used for fluorimetric measurements after hydrolysis (see following discussion).

Compound 3 shows a photobinding to DNA very similar, even if only slightly lower, to that of the parent 2, while clearly lower is that shown by derivative 4. Finally, compound 5 demonstrates a very low photobinding capacity, even lower than that of reference angelicin, 1 (see Figure 2). In comparing the effect of introducing on compound 2 and trioxsalen the same groups on the photobinding to DNA, we observed a somewhat analogous behavior, with an exception concerning cationic derivatives. Both angular and linear compounds, in fact, carrying hydroxymethyl or methoxymethyl groups show a photobinding capacity toward DNA generally lower than the parent compounds. In the case of compounds carrying cationic groups, the angular one shows a strongly decreased photobinding to DNA, while the previously described linear derivative showed an exceptionally increased photoreactivity for the macromolecule.[12]

Evaluation of Cross-linkages in DNA after Irradiation in the Presence of 4,5′-Dimethylangelicin Derivatives 3–5. Angelicins have two different photoreactive sites present in their molecule, and from a photochemical point of view they should be considered bifunctional reagents, such as psoralens; however, it has been clearly shown that in the presence of native DNA under physiological conditions (B form) these substances behave as monofunctional reagents.[6,8] In fact, in the presence of DNA they undergo intercalation between two base pairs and in such a condition, for geometrical reasons, they cannot engage both the photoreactive sites,[6,8,10,11] as occurs for psoralens,[5-7] but only one. For this reason, angelicins cannot form bifunctional linkages.

For a further confirmation of this behavior, we have examined DNA samples irradiated in the presence of the new compounds and compared their renaturation capacity

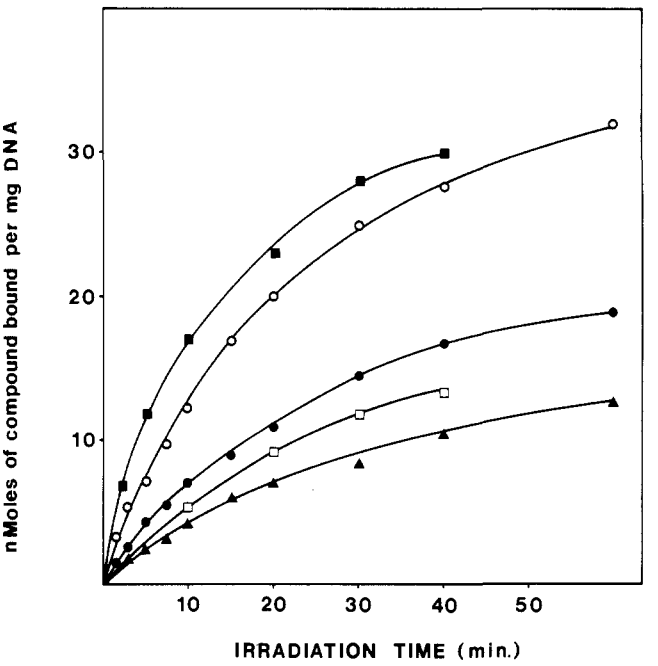

Figure 2. Photobinding capacity shown by 3 (○), 4 (●), and 5 (▲) toward DNA. The photobinding capacities of 1 (□) and 2 (■), as reference compounds, are also reported.

with a DNA sample cross-linked by psoralen to ascertain the absence of interstrand cross-linkages. Cross-linked DNA, in fact, undergoes spontaneous renaturation after heat denaturation, and the extent of renaturation can be evaluated quantitatively by hydroxylapatite chromatography.[11] Elution profiles of the chromatography (see Figure 3) of the samples of DNA irradiated in the presence of the compounds and then heat denatured clearly showed that no renaturation occurred, while DNA samples irradiated in the presence of psoralen for the same time underwent complete renaturation. These data show that no interstrand cross-linkages are formed in the photoreaction between these compounds and DNA.

Fluorimetric Determinations. Monofunctional binding of psoralen to pyrimidine bases of DNA occurs through two different monoadducts: 4′,5′ fluorescent and 3,4 nonfluorescent cycloadducts.[5,6,17-19] Also, compound 2 seems to bind to DNA in similar way; recently, in fact, the 4′,5′ fluorescent cycloadduct between thymine and 2 has been isolated from the products of hydrolysis of DNA irradiated in the presence of 2, and indications of the presence of 3,4 cycloadducts have been obtained.[20]

For having indications on the extent of formation of fluorescent adducts in DNA irradiated in the presence of the new angelicins (i.e., that, other than the methyl group in the 5′ position, have an additional group in the 4′ position), we have evaluated the fluorescence acquired by the macromolecule. In our experiments, the remaining (part B) samples coming from the irradiation of DNA in the presence of 3–5 have been hydrolyzed to avoid fluorescence quenching due to the intercalation of fluorescent cycloadducts in duplex DNA.[21]

(17) L. Musajo, G. Rodighiero, and F. Dall'Acqua, *Experientia*, 21, 24 (1965).
(18) F. Dall'Acqua, S. Marciani, F. Zambon, and G. Rodighiero, *Photochem. Photobiol.*, 29, 489 (1979).
(19) L. Musajo, F. Bordin, G. Caporale, S. Marciani, and G. Rigatti, *Photochem. Photobiol.*, 6, 711 (1967).
(20) F. Dall'Acqua, S. Caffieri, D. Vedaldi, A. Guiotto, and G. Rodighiero, *Photochem. Photobiol.*, in press.
(21) F. Dall'Acqua, S. Caffieri, and G. Rodighiero, *Photochem. Photobiol.*, 27, 77 (1978).

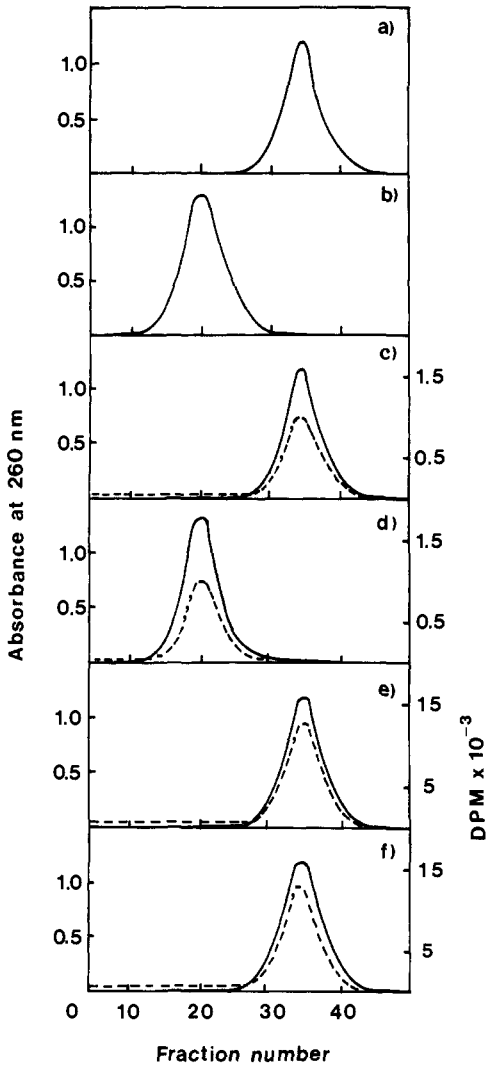

Figure 3. Elution profiles of various DNA samples (2.3×10^{-3} M) having a native double-stranded structure (maxima absorbance of the elution peak at 34th fraction) and denatured single-stranded structure (26th fraction), chromatographed on an hydroxylapatite column and eluted with a linear phosphate buffer gradient.[22] The reference DNA (part a) when heated undergoes irreversible heat-denaturation (part b[6]). When in DNA are present interstrand cross-linkages, such as in the DNA sample irradiated (120 min) in the presence of [^{3}H]psoralen (4.7×10^{-5} M, 1.9 Ci/mol[11]), it cannot undergo irreversible denaturation as shown by the identity of the elution profiles (part e) and after heating (part f).[5-7] DNA sample irradiated (120 min) in the presence of tritiated compound **3** (4.7×10^{-5} M) shows elution profiles before (part c) and after heating (part d) identical with those of reference DNA, evidencing that heat-denaturation occurs and therefore no interstrand cross-linkages are formed in its photoreaction with DNA.[6]

The excitation as well the fluorescence spectra of the hydrolyzed solutions were identical with that of the fluorescent 4',5' cycloadduct between compound **2** and thymine. The fluorescence acquired by DNA after irradiation demonstrates that, in spite of the presence of two groups both in 4' and 5' positions, the 4',5' double bond of the new angelicins may be involved in the monofunctional addition to DNA.

The fluorescence intensity acquired by DNA as a function of the time of irradiation in the presence of the new compounds is reported in Figure 4a. In Figure 4b, the "specific fluorescence", obtained by dividing the fluorescence intensity by the number of nanomoles of the substance covalently linked to 1 mg of DNA after the same irradiation time, is also reported.[22,23]

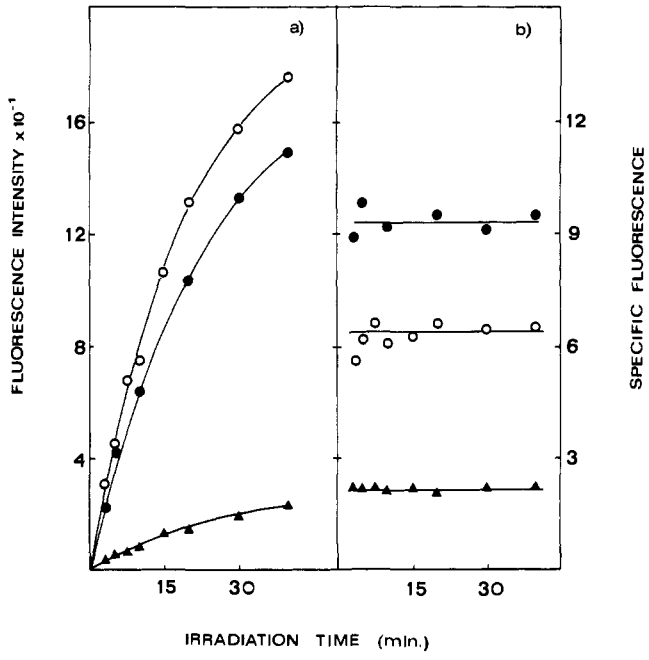

Figure 4. Fluorescence (exciting wavelength, 330 nm; fluorescent wavelength, 390 nm) acquired by DNA under irradiation (365 nm) in the presence of **3** (○), **4** (●), and **5** (▲) after hydrolysis in acidic medium. Part a: fluorescence intensity after irradiation in the presence of equimolecular amounts of compounds. Part b: specific fluorescence obtained by dividing the measured fluorescence intensity value by the amount of substance linked to DNA (nanomoles linked per milligram of DNA).

Considering that both fluorescent and nonfluorescent adducts can be formed in the photoaddition with DNA, "specific fluorescence" can give a better indication on the extent of 4',5' cycloadducts formed than the simple fluorescence intensity.[22,23]

We observed that the specific fluorescence values do not decrease by increasing the time of irradiation, and this fact proves that fluorescent adducts once formed cannot later photoreact further with another pyrimidine base as occurs for linear furocoumarins, which through this second cycloaddition form interstrand cross-linkages.[5,6,18] This is indirect and further evidence that these angelicins cannot form bifunctional adducts.

Taking into account that the groups introduced into the 4' position of **2** should not produce marked electronic perturbation as shown by the UV absorption spectra of the new compounds that are practically superimposable on that of the parent **2**, we can assume that the quantum yield of fluorescence of the three 4',5' cycloadducts derived from the new compounds is similar. On the basis of this assumption, the specific fluorescence data indicate that, in respect to the total photobinding, the extent of 4',5' cycloadduct formation should be the highest for **4**, a little lower for **3**, and even lower for **5**.

Flow Dichroism Measurements. In previous experiments it has been shown that when a furocoumarin forms a complex in the ground state with DNA it undergoes intercalation between two base pairs of duplex DNA.[5-7,13]

To investigate the reason why compound **5**, even if forming effectively a complex with DNA (see Figure 1 and Table II), showed a very low photobinding capacity (see Figure 2), we have examined its complex with native DNA

(22) D. Vedaldi, F. Dall'Acqua, S. Caffieri, and G. Rodighiero, *Photochem. Photobiol.*, **29**, 277 (1979).
(23) F. Dall'Acqua, D. Vedaldi, and M. Recher, *Photochem. Photobiol.*, **27**, 33 (1978).

Table III. Inhibition of DNA and RNA Synthesis in Ehrlich Ascites Tumor Cells by Irradiation (365 nm) in the Presence of Angelicins[a]

no.	D_{50} (quanta × 10^{-18} ± confidence limits)[b] DNA	RNA
1	25.0 ± 1.1	23.3 ± 1.6
2	10.8 ± 1.54	13.5 ± 2.1
3	9.2 ± 1.3	13.8 ± 1.8
4	22.7 ± 2.5	13.8 ± 1.9
5	100	100

[a] The tumor cells were irradiated in the presence of 1.9×10^{-5} M compound and then incubated at 37 °C in the presence of [^{3}H]thymidine or of [^{3}H]uridine; the acid-insoluble radioactivity was determined. The results are expressed as D_{50}, i.e., the UV radiation dose that in the presence of the drug produces a 50% inhibition in respect to untreated cells. Incubation in the dark in the presence of the drugs or irradiation in their absence were both ineffective. [b] $p = 0.05$.

by means of flow dichroism measurements to investigate whether it undergoes intercalation when complexed with the macromolecule. The experiments performed clearly showed an evident negative dichroism in correspondence to the chromophore of **5**; this fact demonstrates that the planar moiety of **5** assumes an ordered position when complexed with DNA and, according to previous findings,[13] the negative sign of dichroism is in favor of the intercalation of this compound between two base pairs of DNA.

Also, the strong fluorescence quenching of this compound when complexed with DNA (quantum yield of **5** complexed with the macromolecule is 30 times lower than that of free **5**) is in favor of intercalation.

Photobiological Effects. To evaluate the skin photosensitizing activity of the new angelicins, we have operated according to the classic test of Musajo et al.[24] Measured volumes of the ethanolic solutions of the three compounds were placed by evaporation of the solvent on a 4-cm^2 square area of depilated albino guinea pig skin up to a total concentration of 50 μg/cm^2; the area was irradiated for 60 min with a HPW 125 Philips lamp, provided with a Philips filter, emitting over 90% at 365 nm light,[25] at a distance of 20 cm. The animals were kept in the dark and observed 12, 24, and 48 h after irradiation. Under these conditions the three substances did not show any capacity to induce skin sensitization.

The inhibition of DNA and RNA synthesis in Ehrlich ascites tumor cells was determined after irradiation of the cells in the presence of the three substances. Using the data of several experiments, we calculated the values of D_{50} (extent of UV-A irradiation dose that in the presence of the drug produces 50% inhibition and that is inversely correlated with photobiological activity), which are reported in Table III. Derivative **3** exhibited a strong activity, strictly similar to that of the parent **2**, while the activity of **4** was clearly lower. Finally, compound **5** showed a very low and not correctly evaluable activity.

The survival curves obtained by irradiating the T_2 phage in the presence of the drugs studied are reported in Figure 6: compound **3** showed a very high photosensitizing activity on the same order of magnitude as **2** (after 10 min of irradiation, the T_2 phage was inactivated), while compound **4** appeared less effective. Finally, compound **5** showed very poor activity, similar to that of the reference, angelicin (**1**); actually even after 20 min of irradiation a large fraction of the phage retained its infectivity.

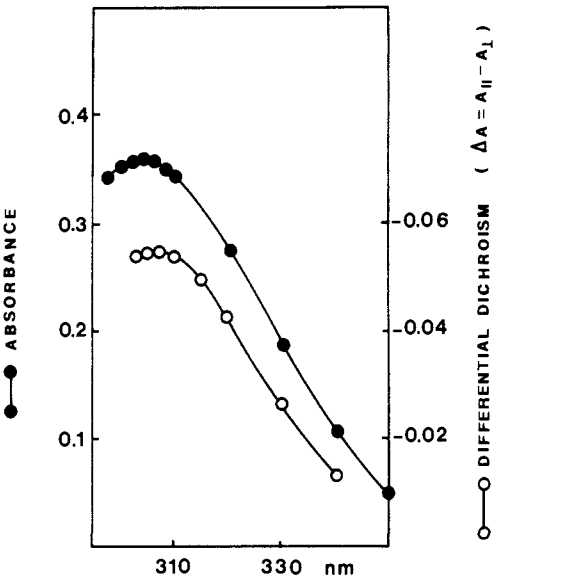

Figure 5. Differential dichroism and UV absorption spectra of compound **5** (6×10^{-4} M) in the presence of calf thymus DNA (1.51×10^{-3} M); optical cells having a 1-mm optical path were used. Velocity gradient of the laminar flow in our experimental conditions was 2900 s^{-1}.[13]

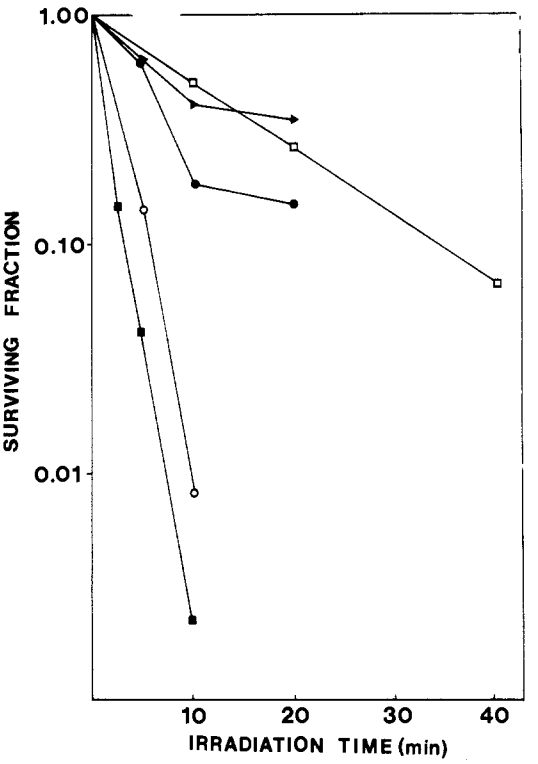

Figure 6. Inactivation of T_2 phage by irradiation (365 nm) in the presence of compounds **3**–**5** and for reference compounds **1** and **2** (1.9×10^{-5} M); symbols as in previous figures. After irradiation, the plaque-forming units per milliliter were scored using *E. coli* B_{48} as indicator bacteria. Incubation in the dark in the presence of the drugs and 40-min irradiation in their absence were both unable to reduce the surviving fraction significantly.

Discussion and Conclusion

The three derivatives of the angular furocoumarin (**2**) in the photoreaction with DNA form only monofunctional adducts, confirming that angular furocoumarins cannot form interstrand cross-linkages in native DNA. These

(24) L. Musajo and G. Rodighiero, *Experientia*, 18, 153 (1962).
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compounds, therefore, as a consequence of their inability to provoke bifunctional lesions in DNA, are not able to induce skin phototoxicity typical of psoralens. In fact, these substances applied on guinea pig skin and irradiated according to the classic test of Musajo et al.[24] were not able to induce skin erythema.

Concerning the complex that they form with DNA in the ground state, we can observe that the three compounds show different affinities for the macromolecule; also, the photobinding to DNA is different for the three derivatives. When the capacity to form the complex remains similar to the parent compound **2**, as for derivative **3**, the successive photoaddition to DNA also takes place in a very similar way; however, when the capacity of the complex formation is markedly reduced, as for **4**, the successive extent of photoaddition is also decreased. These data seem to confirm that the formation of the preliminary complex markedly affects the successive photocycloaddition as previously shown.[6,7,11,13,18]

The unusual behavior shown by **5** of an increased capacity in the dark for complex formation corresponds a strongly decreased photobinding capacity. In this connection, flow dichroism measurements and fluorescence quenching data strongly support the fact that this compound, when complexed with DNA, undergoes intercalation between two base pairs of the macromolecule. On the basis of this assumption, the reduced photobinding to DNA may be explained by assuming that the small distance between the cationic group, which binds externally to the phosphate groups of DNA, and the angular intercalated tricyclic moiety leads to a disposition of this moiety not suitable for the photoaddition.

The fluorescence data show that the 4′,5′ double bond of the new angelicins, in spite of the presence of two groups both in the 4′ and 5′ positions, is involved in the monofunctional cycloaddition to pyrimidine bases of DNA. Assuming that the quantum yield of fluorescence is similar in the three derivatives, the specific fluorescence data indicate that in respect to the total photobinding the highest percentage of fluorescent adducts is formed by compound **4**, that of **3** is a little lower, and that of cationic compound **5** is even lower still.

These angelicins have shown evident photobiological activity both on Ehrlich ascites tumor cells, where DNA and RNA syntheses were strongly inhibited, and also on T_2 phages, where their infectivity was markedly decreased. In these biological systems the highest activity is shown by **3** while those of **4** and **5** were gradually lower.

The data now obtained clearly show that for these new angelicins a close correlation exists between photobinding capacity to DNA and photobiological activity.

In the case of angelicins this correlation is direct because to an extent of monofunctional photodamage corresponds a parallel extent of photobiological effect, while for linear furocoumarins, where in addition to monofunctional lesions bifunctional ones (having much stronger biological consequences) are also formed, a direct correlation between total photobinding and photobiological effects is not always so immediate.

Among the three compounds, **3**, which in addition to a strong photobiological activity that is clearly explicated in terms of inhibition of cell division also shows a complete lack of skin phototoxicity and a very low mutagenic activity,[26] should be considered as a possible drug for the photochemotherapy of psoriasis and other skin diseases characterized by skin hyperproliferation.

Experimental Section

Melting points determined in open capillary tubes are uncorrected. ^{1}H NMR spectra were recorded at 60 MHz on a Hitachi-Perkin Elmer R-24A spectrometer with Me_4Si as the internal standard (δ 0) and coupling constants are given in hertz. The relative peak areas and decoupling experiments were in agreement with all the assignments. UV absorption spectra were determined in ethanolic solutions by means of a Perkin-Elmer instrument, Model 554. All compounds were monitored by TLC using precoated Merck 60-F-254 0.24-mm plates, developed either by chloroform or by ethyl acetate/cyclohexane (1:2). Microanalyses were performed in the analytical laboratory of our Institute by Professor A. Pietrogrande. Where the analyses are indicated by the symbols of the elements, analytical results were within the ±0.4% of the theoretical values. Angelicin (**1**) and 4,5′-dimethylangelicin (**2**) have previously been prepared by chemical synthesis in our Institute. Compounds **1** and **2** have been labeled and purified as described elsewhere.[11] The specific activity of the various labeled compounds is reported in Table I.

Calf thymus DNA (Cat. D 1501) was obtained from Sigma Chemical Co. (St. Louis, Mo); the sample showed a hypochromicity over 40%, determined according to Marmur and Doty.[27] Hydroxylapatite, Bio-Gel type, was furnished by Bio-Rad laboratories (Calif.).

4′-(Chloromethyl)-4,5′-dimethylangelicin (6). Compound **2** (0.75 g, 3.5 mmol) was reacted in an acetic acid solution at room temperature for 48 h with chloromethyl methyl ether (two 7.5-mL portions) according to Isaacs et al.[12] After this time, the chloromethyl methyl ether excess and part of the acetic acid were eliminated under reduced pressure by heating gently. After the mixture was cooled overnight at 5 °C, a crop of white crystals of **6** was obtained (0.580 g, 63%): mp 211–213 °C by recrystallization from acetic acid; ^{1}H NMR ($CDCl_3$) δ 7.40 and 7.34 (2 H, s, 5-H and 6-H), 6.27 (1 H, br s, 3-H), 5.00 (2 H, s, 4′-CH_2Cl), 2.60 and 2.53 (6 H, s, 4-CH_3 and 5′-CH_3). Anal. ($C_{14}H_{11}O_3Cl$) C, H, Cl.

4′-(*N*-Phthalimidomethyl)-4,5′-dimethylangelicin (7). A mixture of 4′-(chloromethyl)-4,5′-dimethylangelicin (**6**; 0.697 g, 2.65 mmol), potassium phthalimide (0.631 g, 3.35 mmol), and *N*,*N*-dimethylformamide (40 mL) was heated at 100 °C for 8 h. The yellow residue, obtained by working up the mixture as described elsewhere,[12] was suspended in dry methanol (50 mL) and the undissolved **7** was obtained by filtration (0.238 g, 24%): mp 273 °C after crystallization from chloroform. ^{1}H NMR ($CDCl_3$) δ 7.76 and 7.70 (4 H, d, phthalimide), 7.36 and 7.32 (2 H, s, 5-H and 6-H), 6.22 (1 H, br s, 3-H), 5.30 (2 H, s, 4′-CH_2N), 2.60 (6 H, s, 4-CH_3 and 5′-CH_3). Anal. ($C_{22}H_{15}NO_5$) C, H, N.

4′-(Aminomethyl)-4,5′-dimethylangelicin Hydrochloride (5). Compound **7** (0.173 g, 0.46 mmol) was hydrolyzed by hydrazine hydrate.[12] The crude **5** (0.079 g, 61%) was dissolved in absolute ethanol (10 mL) and dry ether was added to thickening (10 mL); by standing and cooling a crop of white needles was obtained (0.048 g): mp 302 °C; UV (95% EtOH) λ_{max} 298 nm (log ϵ 3.92), 262 (sh, 4.23), 251 (4.37); λ_{min} 273 nm (log ϵ 3.67), 229 (3.96); ^{1}H NMR (D_2O) δ 7.35 and 7.32 (2 H, s, 5-H and 6-H), 6.14 (1 H, br s, 3-H), 4.20 (2 H, s, 4′-CH_2NH_2), 2.50 and 2.38 (6 H, s, 4-CH_3 and 5′-CH_3). Anal. ($C_{14}H_{14}ClNO_3$) C, H, N.

4′-(Hydroxymethyl)-4,5′-dimethylangelicin (3). A suspension of **6** (0.240 g) was hydrolyzed in an aqueous (100 mL) solution.[12] The cooled solution was extracted with ethyl acetate. From the organic solution, dried on Na_2SO_4, the solvent was distilled, and the residue obtained was crystallized from ethyl acetate to give **3** (0.138 g, 62%): mp 201 °C; UV (95% EtOH) λ_{max} 299 nm (log ϵ 3.96), 262 (sh, 4.26), 253 (4.38); λ_{min} 276 nm (log ϵ 3.71), 231 (3.88); ^{1}H NMR ($CDCl_3$) δ 7.26 and 7.22 (2 H, s, 5-H and 6-H), 6.21 (1 H, br s, 3-H), 4.92 (2 H, s, 4′-CH_2OH), 2.58 and 2.52 (6 H, s, 4-CH_3 and 5′-CH_3). Anal. ($C_{14}H_{12}O_4$) C, H.

4′-(Methoxymethyl)-4,5′-dimethylangelicin (4). Compound **6** (0.200 g) was refluxed in MeOH (50 mL) solution for 2 h.[12] The solvent was removed and the residue was crystallized from MeOH

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to give 4 (0.141 g, 72%): mp 137 °C; UV (95% EtOH) λ_{max} 298 nm (log ϵ 3.93), 262 (sh, 4.25), 252 (4.40); λ_{min} 275 nm (log ϵ 3.70) 230 (3.90); ^{1}H NMR ($CDCl_3$) δ 7.36 and 7.30 (2 H, s, 5-H and 6-H), 6.19 (1 H, br s, 3-H), 4.75 (2 H, s, 4'-CH_2O), 3.48 (3 H, s, OCH_3), 2.56 and 2.48 (6 H, s, 4-CH_3 and 5'-CH_3). Anal. ($C_{15}H_{14}O_4$) C, H.

Complexes in the Ground State. Binding experiments were performed in aqueous solutions containing 1.5×10^{-2} M NaCl and 1×10^{-3} M EDTA. Dialysis experiments,[13] as well as fluorimetric titrations,[14] are described elsewhere.

Computation of the Interaction Parameters. The method of computation involved an iterative procedure designated to satisfy the following equation of Mc Ghee and Von Hippel:[16]

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

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 the 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.

Irradiation Procedure. To aqueous solution (containing a 2×10^{-3} M NaCl) of DNA (2.3×10^{-3} M) was added of 4.7×10^{-5} M compound to be examined; the concentration of the angelicins was always checked by radiochemical measurements and, if necessary, was corrected. Irradiation was made in a test tube immersed in a thermostatically controlled cell; the irradiation intensity, determined by means of a chemical actinometer,[28] was 1.07×10^{16} quanta s^{-1} mL^{-1}. After irradiation, solid NaCl was added until 2 M and 2 volumes of absolute ethanol were added; precipitated DNA, collected by centrifugation, was washed with 80% ethanol and redissolved in the initial volume of water.

Radioactivity Measurements. A liquid scintillation spectrometer (Packard Model 3375) was employed. Dioxane base (PPO, 5 g; POPOP, 0.075 g; naphthalene, 120 g; dioxane, up to 1000 mL of solution) or toluene base (PPO, 5 g; POPOP, 0.05 g; toluene, up to 1000 mL of solution) scintillators were used.

Evaluation of Cross-linkages. This evaluation was made directly on the irradiated DNA samples, without precipitation, accordingly to reference procedures.[22]

Fluorimetric Measurements. Fluorimetric measurements were carried out on part B of the irradiated samples after acidic hydrolysis,[21] by means of a spectrophotofluorimeter (Perkin-Elmer, Model MPF-044).

Flow Dichroism Measurements. Flow dichroism measurements were carried out by means of a Schimadzu QV-50 spectrophotometer with a flow dichroism attachment, which replaces the usual cell chamber and consists of a quartz cylindrical cell containing a quartz rotating cylinder and a calcite prism that polarizes the monochromatic light parallel (||) and perpendicular ($\perp$) to the flow line.[29] Linear flow dichroism as well as the reduced dichroism were calculated according to Wada[30] and Wada and Kozawa.[29]

DNA and RNA Synthesis. Ehrlich ascites tumor cells in Hank's solution (aliquots of 5 mL; 2×10^7 cells/mL) were irradiated in petri dishes (5 cm in diameter) placed on ice by a Philips HPW 125 set at 20-cm distance (2.02×10^{16} quanta/s incident on the whole sample). Samples of 2×10^6 of the irradiated cells in 0.5 mL in the same medium containing 1 μCi of [^{3}H]thymidine or of [^{3}H]uridine were incubated for 15 min at 37 °C. The reaction was stopped by chilling on ice and adding 1 mL of 5 mM unlabeled nucleoside. The cells were collected by filtering on Whatman CF/c dishes (diameter 2.5 cm), washed three times with saline, and treated with 10mL of ice-cold 10% trichloroacetic acid. After 1 min, the samples were filtered and washed six times with 10 mL of 10% trichloroacetic acid; the filters were dried and counted. Each UV-A radiation dose was studied at least in duplicate, while the controls were four samples of untreated cells. Results were calculated on the basis of the specific radioactivity incorporated into nucleic acids and expressed as percentage of the incorporation observed in the untreated cells (2000 dpm/μg of DNA and 2400 dpm/μg of RNA). DNA and RNA contents were determined by the diphenylamine[31] and orcinol[32] reactions. The data were submitted to probit analysis and expressed as the D_{50}, i.e., the UV-A radiation dose that in the presence of the 1.9×10^{-5} M produces a 50% inhibition.

T_2 Phage Inactivation. T_2 phage was grown using *Escherichia coli* B_{48} as host bacteria and the brain-heart infusion broth (from Difco Laboratories, Mich.); virions were irradiated in Hank's 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.

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Potential Inhibitors of Nucleotide Biosynthesis. 1. Nitrosoureidonucleosides. 2

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Several nitrosoureidonucleosides (**9a**, **9b**, **11a**, **11b**, 18 and **20**) designed as inhibitors of enzymes that metabolize purine and pyrimidine nucleotides have been prepared and their chemical and biological properties studied. The low level of biological activity observed may be due to the unexpected stability of these nitrosoureas compared to biologically active compounds such as *N*-methyl-*N*-nitrosourea (MNU), *N*,*N*'-bis(2-chloroethyl)-*N*'-nitrosourea (BCNU), and *N*,*N*'-dicyclohexyl-*N*-nitrosourea (DCyNU).

Except for the early reactions of the biosynthetic pathway to uridylic acid, all of the enzymatic reactions leading to ribonucleotides of RNA and the 2'-deoxyribonucleotides of DNA take place at the mono- and diphosphate levels.[1,2] The phosphate moieties of the compounds involved are important to their binding to the active site of the enzymes that carry out these transformations. Almost all of the known inhibitors of these enzymes are nucleoside mono- and diphosphates. These compounds, however, are labile

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