

## 6-Methylangelicins: A New Series of Potential Photochemotherapeutic Agents for the Treatment of Psoriasis

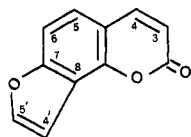
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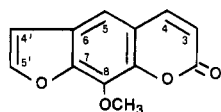
The possible presence of methylpsoralens as undesired inquinants in synthetic methylangelicins has been avoided through a synthetic pathway starting from umbelliferones carrying a methyl group in the 6-position. The new 6-methylangelicins show a high affinity toward DNA, forming in the dark a molecular complex; the complexed angelicins under UV-A irradiation photobind effectively to the macromolecule, forming only monoadducts. The new compounds show an evident antiproliferative activity by inhibiting DNA synthesis on Ehrlich cells; great differences, however, can be seen between the various compounds. All the compounds are lacking of skin erythemogenic activity. Some of the new 6-methylangelicins, evaluated in terms of mutagenic activity, demonstrate to be less effective than 8-methoxypsoralen (8-MOP), used for a comparison. On the basis of antiproliferative activity, lack of skin phototoxicity, and low mutagenicity, two compounds have been chosen for clinical evaluation. The compounds tested on seven psoriatic patients by topical application and UV-A irradiation proved to be more effective than 8-MOP, used in the same conditions.

Recently we have prepared and studied various methylangelicins with the aim of obtaining new potential agents for the photochemotherapy of skin diseases characterized by hyperproliferation, e.g., psoriasis and mycosis fungoides,<sup>1-5</sup> and for treatment of pigmentation disorders (vitiligo).<sup>1</sup>

While methylangelicins realize their antiproliferative activity photoinducing in the cell DNA only monofunctional lesions,<sup>2-6</sup> psoralens such as 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), 4,5',8-trimethylpsoralen (TMP), that is, the classic agents used for photochemotherapy, called also PUVA (psoralen plus ultraviolet-A radiation) therapy, also photoinduce bifunctional lesions (interstrand cross-linkages);<sup>7</sup> to these latter lesions are mainly ascribed some undesired side effects of PUVA therapy such as genotoxicity and the consequent risk of skin cancer and skin phototoxicity.<sup>8,9</sup>



angelicin

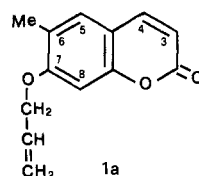


8-methoxypsoralen (8-MOP)

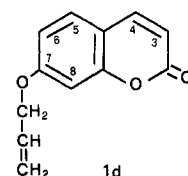
Methylangelicins show evident antiproliferative activity, a genotoxicity lower than that shown by psoralens, and, in general, a lack of skin phototoxicity;<sup>1,5,6</sup> the only exception in this connection is represented by the 4'-methylangelicins which show skin phototoxicity to a measurable extent.<sup>5,10</sup>

The synthetic pathway for preparing methylangelicins starts from *O*-allylumbelliferone (**1d**) (or from its methyl derivatives), which by transposition of the allyl group leads to the 8-allylumbelliferones accompanied by a lesser amount of the corresponding 6-allyl isomers.<sup>4</sup> The separation of these two isomers, carried out by means of column chromatography, must be complete because during the subsequent synthetic steps the 6-allylumbelliferones lead to the formation of methylpsoralens which are very ef-

fective in inducing bifunctional photolesions to the DNA.<sup>7,11</sup>



1a



1d

With the aim of avoiding this time-consuming step and of obtaining methylangelicins assuredly lacking in psoralens, we planned on preparing a new series of methylangelicins starting from 6-methylumbelliferones (**1a-c**); in such a way the undesired 6-allyl intermediates cannot be formed and no trace of psoralens can be present in the final methylangelicins.

In this paper we describe the preparation of a new series of 6-methylangelicins, their interactions with DNA, and their antiproliferative skin phototoxic and genotoxic activity. Finally, preliminary clinical data concerning the

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- (4) Guiotto, A.; Rodighiero, P.; Pastorini, G.; Manzini, P.; Bordin, F.; Baccichetti, F.; Carlassare, F.; Vedaldi, D.; Dall'Acqua, F. *Eur. J. Med. Chem.* 1981, 16, 489.
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- (6) Dall'Acqua, F. In "Trends in Photobiology"; Hélène, C., Charlier, M., Montenay-Garestier, Th., Laustriat, G., Eds.; Plenum: New York, 1982; p 267.
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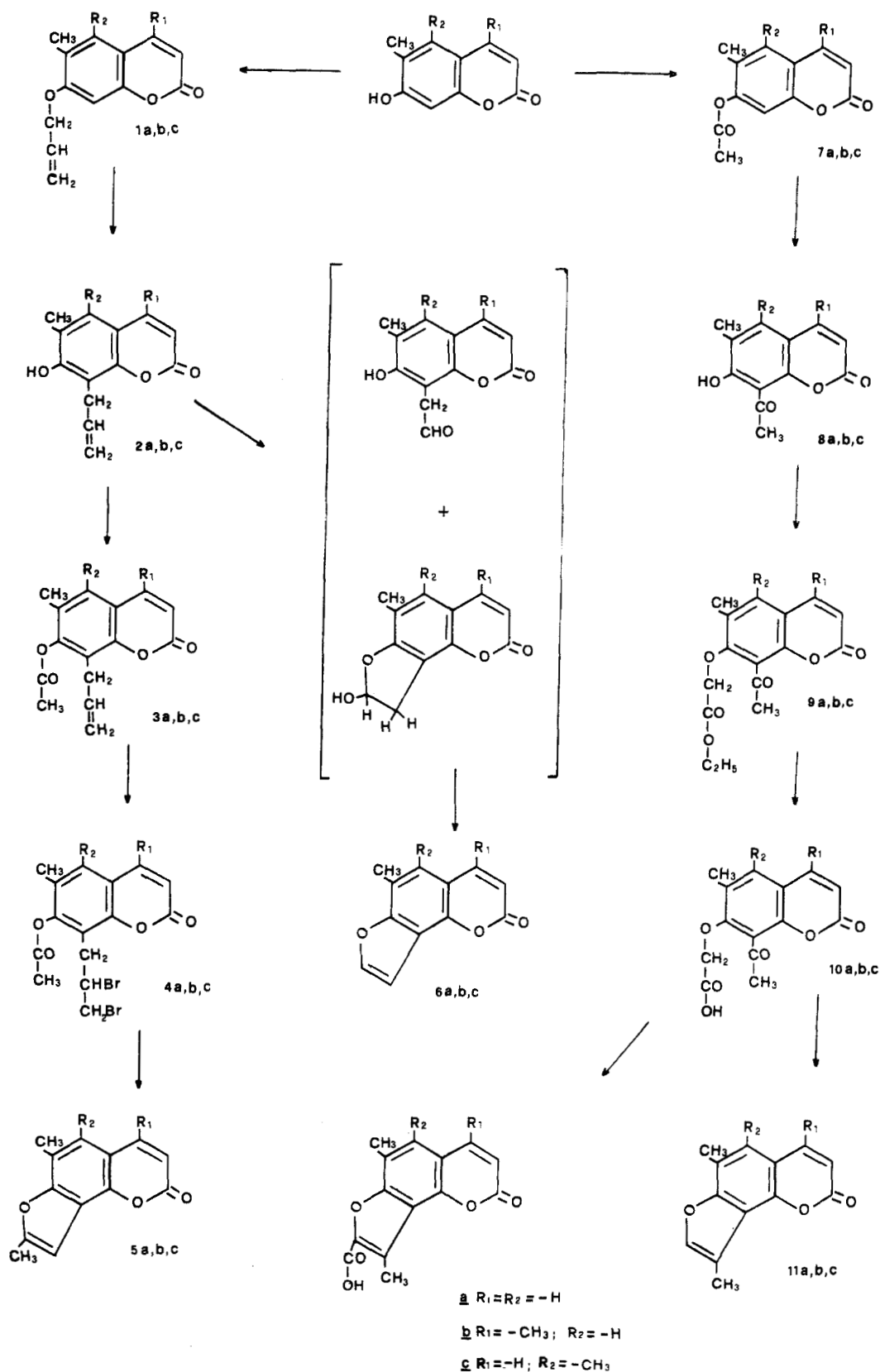
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Scheme I



capacity of these compounds to clear up psoriatic lesions are reported.

### Results

**Chemistry.** The starting products were 6-methylumbelliferone (6-methyl-7-hydroxycoumarin) or its 4- and 5-methyl derivatives (see Scheme I). From these 7-hydroxycoumarins the corresponding 7-allyl ethers have been prepared (1a-c); by Claisen rearrangement of the allyl

group, these latter compounds furnished the 8-allyl-7-hydroxycoumarins. Methylangelicins carrying a methyl group in the 5'-position, such as the original 6,5'-dimethylangelicin (5a), 4,6,5'-trimethylangelicin (5b), and 5,6,5'-trimethylmethylangelicin (5c) were prepared according to the Kaufman procedure;<sup>12</sup> in this way the proper

(12) Kaufman, K. D. *J. Org. Chem.* 1961, 26, 117.

**Table I.** Binding Parameters of the Complexes between 6-Alkylangelicins and DNA and Rate Constants of Their Photoreactions with the Macromolecule

compd	<i>K</i>	<i>n</i> <sup>a</sup>	1/ <i>n</i> <sup>b</sup>	rate constant, min <sup>-1</sup>
angelicin (1)	560	15.87	0.063 ± 0.003	1.10 × 10 <sup>-2</sup>
6-methylangelicin (6a)	925 ± 64	20.83	0.048 ± 0.002	3.60 × 10 <sup>-2</sup>
4,6-dimethylangelicin (6b)	2990 ± 232	15.87	0.063 ± 0.003	5.23 × 10 <sup>-2</sup>
6,4'-dimethylangelicin (11a)	6300 ± 541	16.39	0.061 ± 0.003	9.90 × 10 <sup>-2</sup>
6,5'-dimethylangelicin (5a)	1975 ± 136	12.82	0.078 ± 0.004	3.00 × 10 <sup>-2</sup>
4,6,4'-trimethylangelicin (11b)	10100 ± 1190	10.99	0.091 ± 0.0045	13.30 × 10 <sup>-2</sup>
4,6,5'-trimethylangelicin (5b)	3310 ± 281	22.22	0.045 ± 0.002	7.02 × 10 <sup>-2</sup>
5,6,5'-trimethylangelicin (5c)	3730 ± 339	14.29	0.070 ± 0.0035	2.30 × 10 <sup>-2</sup>
8-MOP <sup>c</sup>	736 <sup>c</sup>	7.81 <sup>c</sup>	0.128 <sup>c</sup>	3.10 × 10 <sup>-2</sup>

<sup>a</sup> According to McGhee and Von Hippel,<sup>15</sup> *n* is defined here as the number of nucleotides occluded by one bound molecule of angelicin derivative. <sup>b</sup> 1/*n* defines, according to McGhee and Von Hippel, the frequency of binding sites; in other words, it is the number of ligands bound per nucleotide and can be considered analogous to the "*n*" value obtained by the classic Scatchard method.<sup>31</sup> <sup>c</sup> As reference compound; data taken from ref 19.

acetylated 8-allylumbelliferones were brominated and the corresponding 8-dibromopropyl derivatives cyclized in alkaline medium to the above-mentioned 5'-methylangelicins.<sup>4</sup>

Methylangelicins without a methyl group in the furan ring, such as the original 6-methylangelicin (6a), 4,6-dimethylangelicin (6b), and 5,6-dimethylangelicin (6c), were obtained by cyclization, in the presence of phosphoric acid, of the 8-coumarinylacetaldehydes, obtained by ozonolysis of suitable 8-allylumbelliferones.

Methylangelicins carrying a methyl group in the 4'-position, such as the original 6,4'-dimethylangelicin (11a), 4,6,4'-trimethylangelicin (11b), and 5,6,4'-trimethylangelicin (11c), were prepared according to the Metha procedure,<sup>13</sup> in this way the proper 7-*O*-acetoxycoumarins were submitted to the Fries rearrangement, obtaining the 8-acetylumbelliferones, which were condensed with ethyl bromoacetate, and the resulting (7-coumarinyloxy)acetates were hydrolyzed to give the corresponding free acids. By cyclization of the latter compounds, accompanied by an almost complete decarboxylation, the above-mentioned 4'-methylangelicins were obtained. All the 6-methylangelicins are new compounds; in addition, the intermediates are new compounds, except for the 6-methyl-7-acetoxy-coumarin (7a).<sup>14</sup>

#### Molecular Complex in the Ground State with DNA.

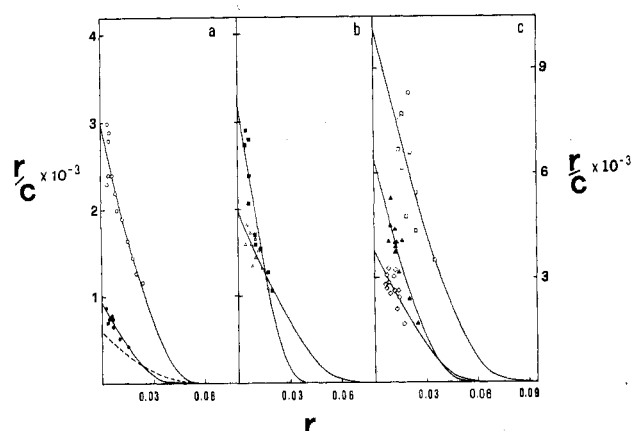
The mechanism of the antiproliferative activity of angelicins is connected with their capacity to induce monofunctional photolesions to the cell DNA.<sup>6</sup> This photochemical event is realized in two steps: the formation of a preliminary complex in the ground state between the furocoumarin and the macromolecule, where the furocoumarin undergoes intercalation between two base pairs of the macromolecule.<sup>3,6</sup> The steric arrangement of the complexed furocoumarin favors its successive photoconjugation with the macromolecule by UV-A irradiation.<sup>5-7</sup>

In a way similar to the other angelicins, the 6-methyl congeners also form a molecular complex with the DNA as evidenced by the modification of the UV absorption properties and by the quenching of the fluorescence.<sup>5</sup>

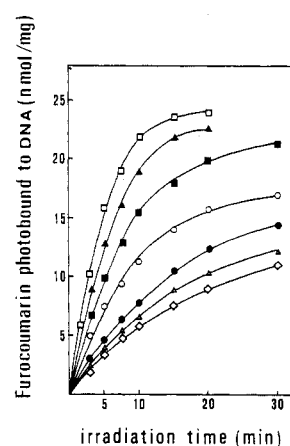
The binding parameters of the complex have been determined by equilibrium dialysis experiments using tritiated angelicins as previously described.<sup>5</sup>

The binding data *r* (molecules of ligand bound per nucleotide) and *c* (ligand free in the system, moles/liter) are reported in Figure 1 according to Scatchard: that is, plotting *r/c* against *r*.

The binding isotherm reported in Figure 1 and the binding parameters, i.e., *K* (association constant to an



**Figure 1.** Scatchard plots for the binding in the dark of 6b (O), 6a (●), and 1 (dotted line), as reference compound (part a), 5b (■) and 5a (Δ) (part b), 11b (□), 11a (▲) and 5c (◇) (part c) to calf thymus DNA. The curves have been calculated by a computer according to the method of McGhee and Von Hippel<sup>15</sup> on the basis of the experimental values of *r* and *c* reported in the figure.



**Figure 2.** Photobinding capacities toward DNA shown by the new 6-methylangelicins. Symbols as in Figure 1.

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 McGhee and Von Hippel<sup>15</sup> on the basis of the experimental data of *r* and *c*.

In general, we can observe that the introduction of one or more methyl groups into the molecule of angelicin leads

(13) Metha, K. H. *Indian J. Chem. Soc.* 1970, 47, 1211.

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to an increase of the affinity to DNA for the dark complex formation in comparison with the parent compound.

As shown by previously prepared methylangelicins,<sup>3,5</sup> also for these new compounds the increased affinity toward DNA as the number of methyl groups increases (see Table I and Figure 1) should be mainly due to the increased hydrophobicity of the parent molecule. In fact 6-methylangelicin (6a) form the dark complex more effectively than parent angelicin 1. The introduction of a second methyl group in the 6-methylated derivative provokes a further strong increase of DNA binding in the dark (see Figure 2 and Table I). This increase is, however, strongly affected by the position of the second methyl group as shown by the order of efficacy of the position, which is the following: 4' > 4 > 5'.

Finally, when a third methyl group is introduced into the dimethyl derivatives 6b, 11a, and 5a, a further increase of DNA binding capacity is observed.

**Photobinding to DNA.** In a way strictly similar to the other methylangelicins, the new 6-methyl derivatives too can photoinduce only monofunctional lesions to the DNA, as shown by the inability of the DNA irradiated in their presence to undergo renaturation after heat denaturation.<sup>3,5,6</sup>

The photobinding of the new angelicins to DNA, determined as previously described,<sup>3</sup> is reported in Figure 2. These photoreactions also behave as pseudo-first-order reactions with respect to the complexed furocoumarin to the macromolecule.<sup>3</sup> The rate constant values are reported in Table I.

The introduction of a methyl group in the 6-position markedly enhances the DNA photobinding in comparison with the parent angelicin.

Taking into account previous photobinding data of various monomethyl derivatives of angelicin, the role of the position in terms of DNA photobinding has the following order of importance: 4' > 6 > 5 > 5' > 4.

The introduction into the 6-methylangelicin of a second methyl group in the 4- or 4'-position (compounds 6b, 11a) leads to a further increase of the photobinding to DNA, while methylation of the 5'-position (compound 5a) leads to a decrease of the DNA photobinding.

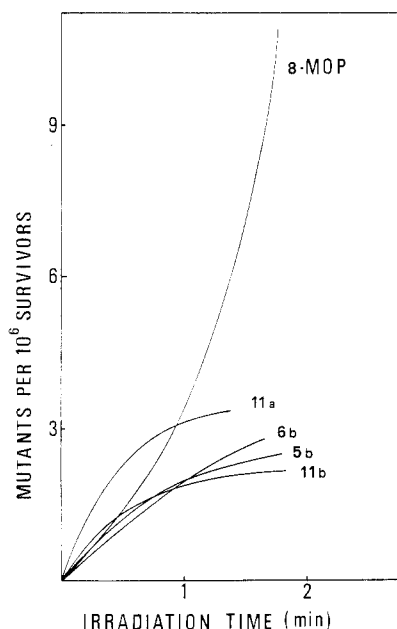
The variation in the DNA photobinding does not correspond to the simple addition of the effects of the methyl groups evaluated separately: e.g., the DNA photobinding of 4,6-dimethylangelicin (6b) should be expected to be lower than that of the 6,5'-dimethyl isomer (5a) while, experimentally, it appears to be the reverse. This is in line with previous observations.<sup>3</sup> The introduction of a third methyl group in the 4- or 4'-position leads to a further enhancement of the DNA photobinding (compounds 11b and 5b), while the methylation in the 5-position of 6,5'-dimethylangelicin provokes a further decrease of the photoaffinity toward DNA.

**Antiproliferative Activity.** The antiproliferative activity of the compounds was determined in terms of their capacity to inhibit DNA synthesis in Ehrlich ascites tumor cells irradiated in their presence.<sup>16</sup> The ID<sub>50</sub> values (irradiation dose which in the presence of the compound provokes the 50% inhibition of the synthesis of the macromolecule), inversely correlated with the antiproliferative activity, are reported in Table II. Except for 6-methylangelicin (6a) and 5,6,5'-trimethylangelicin (5c), which show an antiproliferative activity lower than that of angelicin (1), the other compounds examined (see Table

**Table II.** Inhibition of DNA Synthesis in Ehrlich Ascites Tumor Cells by Irradiation (365 nm) in the Presence of Angelicins and 8-MOP as Reference Compound<sup>a</sup>

compd	ID <sub>50</sub> (quanta × 10 <sup>-18</sup> ± SD; p = 0.05)
angelicin	25.0 ± 1.10
6-methylangelicin (6a)	26.3 ± 0.64
4,6-dimethylangelicin (6b)	4.0 ± 0.59
6,4'-dimethylangelicin (11a)	2.2 ± 0.30
6,5'-dimethylangelicin (5a)	11.86 ± 0.34
5,6-dimethylangelicin (6c)	30.00
4,6,4'-trimethylangelicin (11b)	0.06 ± 0.017
4,6,5'-trimethylangelicin (5b)	3.87 ± 0.86
5,6,5'-trimethylangelicin (5c)	30.00
8-MOP	13.80 ± 0.6

<sup>a</sup> The tumor cells were irradiated in the presence of  $1.9 \times 10^{-5}$  M of the compound studied and then incubated at 37 °C in the presence of [<sup>3</sup>H]thymidine; the acid-insoluble radioactivity was determined. The results are expressed as ID<sub>50</sub>, i.e., the UV radiation dose that in the presence of the drug produces a 50% inhibition with respect to untreated cells. Incubation in the dark in the presence of the drugs or irradiation in their absence were both ineffective.



**Figure 3.** Mutagenic activity in *E. coli* WP2 uvr A by UV-A irradiation in the presence of 11a, 6b, 5b, 11b and of 8-MOP, chosen as a reference compound. The results are reported as regression curves calculated on the basis of three different experiments, according to the following equations: for 8-MOP,  $y = 0.155 + 0.021X + 0.0006X^2$ , SD = 0.34; for 11a,  $y = -0.053 + 0.121X - 0.001X^2$ , SD = 0.11; for 6b,  $y = -0.437 + 0.037X - 0.0001X^2$ , SD = 0.04; for 5b,  $y = 0.083 + 0.044X - 0.0002X^2$ , SD = 0.05; for 11b,  $y = 0.09 + 0.046X - 0.003X^2$ , SD = 0.21. The curves are reported in the range of irradiation times in which significant data have been obtained.

II) exhibit a very strong activity, higher than that of 8-methoxypsoralen (8-MOP).

**Skin Phototoxicity.** The evaluation of skin phototoxicity has been carried out according to Bordin et al.<sup>2,10</sup> Angelicin (50 µg/cm<sup>2</sup>) was applied on to the depilated skin of an albino guinea pig and the area was irradiated with UV-A for 1 h.

Under these conditions the new compounds did not show any erythemogenic activity.

**Genotoxicity.** One of the side effects of the photochemotherapy with psoralens is the risk of skin cancer.<sup>9</sup> Taking into account that a correlation exists between this property and the mutagenic activity of furocoumarins,<sup>17</sup>

(16) Dall'Acqua, F.; Vedaldi, D.; Caffieri, S.; Guiotto, A.; Rodighiero, P.; Baccichetti, F.; Carlassare, F.; Bordin, F. *J. Med. Chem.* 1981, 24, 178.

**Table III.** Results of Topical Photochemotherapy with 6-Methylangelicins (6-MA) in Psoriatic Patients

no.	name	sex	6-MA	maxima dose of UV-A, J/cm <sup>2</sup>	no. of treatments	% clearance		erythema induction <sup>c</sup>		pigmentation capacity <sup>c</sup>	
						6-MA	8-MOP	6-MA	8-MOP	6-MA	8-MOP
1	P.G.	M	11a	8	15	100	85	-	-	+	++
2	M.S.	F	11a	9	15	90	70	-	+	+	++
3	B.M.	F	11b	8	11	100	75	-	+	+++	+
4	U.A.	F	11b	4	11	95		-	+++ <sup>a</sup>	+	-
5	D.B.V.	F	11b	10	15	90	90	-	+++	++	+
6	F.B.	F	11b	6	9	100	80	-	+++ <sup>b</sup>	+	+
7	C.M.P.	F	11b	6	9	100	85	-	++	+	++

<sup>a</sup> The treatment with 8-MOP was stopped after four applications for severe bullous reaction. <sup>b</sup> The treatment with 8-MOP was stopped after five applications because of severe erythema. <sup>c</sup> (+++) very strong, (++) strong, (+) moderate, (-) absent.

we have evaluated the capacity of the more active 6-methylangelicins (6b, 11a, 5b, and 11b) to induce mutants under UV-A irradiation in *Escherichia coli* WP2 uvrA, a strain defective in DNA repair.

The results obtained with the four angelicins and with 8-MOP, chosen as a reference compound, are reported in Figure 3; the mutagenic activity of angelicins appears markedly lower than that of 8-MOP. It should be pointed out that the bacterial strain used, i.e., the uvr-type deficient in DNA repair system, is more sensitive to monoadducts than to diadducts (interstrand cross-linkages formed by psoralens). Bifunctional lesions, in fact, evidence in this strain mainly lethality.<sup>18</sup>

Considering that these four angelicins show an affinity toward DNA in the dark much more pronounced than 8-MOP ( $K = 760$ ),<sup>19</sup> we have endeavored to evaluate whether these angelicins, analogously to 8-MOP, are able to evidence mutagenic activity in the dark. In fact, the extent of mutagenic activity shown by 8-MOP in these conditions is very low and can be observed only with use of a suitable biological substrate.<sup>20</sup> The four angelicins tested in similar conditions, that is on *Salmonella typhimurium* TA 98 strain, in the concentration range between 5 and 20  $\mu\text{g/mL}$  did not show any mutagenicity. Nor was any mutagenic activity observed with the same compounds on TA 100 strain, after metabolic activation with S9 Mix.<sup>21</sup> These data indicate that the use of these compounds without irradiation with UV-A light should not involve risk of genotoxicity. Taking into account that for 8-MOP the induction of frameshift mutations in the dark<sup>20</sup> does not involve damage to DNA<sup>22</sup> and that this property should not involve risk of carcinogenic effect,<sup>22,23</sup> for the 6-methylangelicins now examined, their use without UV-A irradiations should not involve carcinogenic risk.

### Clinical Data

The therapeutical effectiveness of two of the new methylangelicins, that is, 6,4'-dimethylangelicin (11a) and 6,4,4'-trimethylangelicin (11b), which have been chosen from among the various 6-methyl derivatives on the basis

of their antiproliferative activity, of their relatively low genotoxicity, and of their absence of skin phototoxicity on guinea pig skin, were tested by measuring their capacity to clear up psoriatic lesions in various patients (see Table III).

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

In the treated patients, various areas  $4 \times 4$  cm of the affected skin were used: (a) in the first area, an ethanolic solution (0.1% w/v) of the compound under investigation was applied until a concentration of 5  $\mu\text{g/cm}^2$  was reached, and it was left to evaporate by the heat of the body (or by hot air stream). After 20 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<sup>2</sup>; in particular, the initial dose was 2.5 J/cm<sup>2</sup>, and this was gradually increased until 13 J/cm<sup>2</sup> was reached, on the basis of the skin tolerance. (b) In a second area, an ethanolic solution of 8-methoxypsoralen (8-MOP) was applied to a concentration of 5  $\mu\text{g/cm}^2$ , and the area was irradiated in the same way as in area a. (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 the two 6-methylangelicins was repeated generally 5 times a week for 2 or 3 weeks. A good clearing of the psoriatic lesions was generally observed after nine treatments, while in the case of 8-MOP for the same treatments the extent of clearing up was clearly lower. The area treated with the angelicin derivatives but not with UV-A did not show any improvement, while the area treated with UV-A alone showed a very low improvement.

While 11a induced an evident dark pigmentation in the treated area, less pronounced than that induced by 8-MOP in the same conditions, 11b appeared more active (Table III).

The antiproliferative activity has been documented by histological examinations of biopsical specimens carried out on the tested areas, soon after the treatment and one month later.

### Conclusions

The use of *O*-allylumbelliferones methylated in the 6-position as starting material for the synthesis of methylangelicins allows the avoidance of the time-consuming step concerning the purification of 8-allylumbelliferones from their 6-isomers and the obtainment of methylangelicins without any trace of undesired corresponding methylpsoralens.

The new 6-methylangelicins obtained show, in general, a high DNA photobinding and a corresponding very ef-

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fective antiproliferative activity, a genotoxicity lower than that of 8-MOP, and lack of any skin erythemogenic activity.

In this connection, while the first series of methylangelicins (4-, 5-, 5', 4,5', and 5,5'-dimethyl derivatives) showed an antiproliferative activity similar to that of 8-MOP and did not show any skin erythemogenic activity,<sup>3</sup> the second series of compounds, that is, the 4'-methylangelicins, showed a very strong increase of the antiproliferative activity, much higher than that of 8-MOP, accompanied, however, by a certain ability to photoinduce the erythema, especially on human skin.<sup>5,10</sup>

The third series of methylangelicins prepared, the 6-methyl derivatives, represent an evident improvement with respect to the previous congeners; they, in fact, show a very high antiproliferative activity (the highest observed until now in this class of compounds) but lack of any skin erythemogenic activity even on human skin. 6,4'-Dimethyl- (11a) and 4,6,4'-trimethylangelicin (11b), tested clinically on seven patients to evaluate their capacity to clear up psoriasis, proved to be more active than 8-MOP; they did not show any induction of skin erythema but induced an evident dark skin pigmentation. This is a new observation as, in general, skin pigmentation was induced by furocoumarins able also to induce the skin erythema (psoralens<sup>6,24</sup> and 4'-methylangelicins<sup>5,10</sup>).

The capacity of 11a and 11b to induce dark pigmentation on human skin suggested their possible use for the treatment of skin-pigmentation disorders, such as vitiligo, which are commonly cured by topical or systemic PUVA. This possibility is now under investigation by topical treatment of the leukodermic spots of one patient with 11b in combination with UV-A light.

On the other hand, the very high antiproliferative activity of these compounds seems to allow a reduction of the total cumulative UV-A dose (shorter exposure times) for the therapeutic treatment, while their topical use, because of the lack of erythemogenic activity, avoids any negative systemic effects present in the PUVA therapy, such as the immunosuppressive effects, and renders these compounds easier to handle.

Moreover, the topical use of the two above-mentioned angelicins allows the treatment of the affected areas of the skin (e.g., folds, scalp) as well as chronic resistant lesions (e.g., pustular psoriasis, palmoplantaris) where the systemic PUVA treatment is, in general, not effective. The genotoxicity lower than that of 8-MOP may suggest a lower risk of skin cancer as a long-term toxic effect.

These compounds represent new effective potential drugs for the photochemotherapy of psoriasis. Further toxicological and clinical studies are, however, required.

## Experimental Section

Melting points were determined with a Büchi-Tottoli SPM-20 capillary melting point apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was performed on precoated silica gel plates 60-F-254 (Merck; 0.25 mm), developing with a EtOAc-cyclohexane mixture (35/65). Preparative column chromatography was performed with silica gel (Merck; 0.063–0.200 mm). <sup>1</sup>H NMR spectra were recorded on a Varian FT-80 A spectrometer with Me<sub>4</sub>Si as internal standard ( $\delta$  = 0) and CDCl<sub>3</sub> 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 gave satisfactory elemental analyses (within  $\pm 0.3\%$ ). UV spectra were recorded on a Perkin-Elmer Model 554 spectrometer.

**Methylumbelliferone 7-O-Allyl Ethers.** A Me<sub>2</sub>CO (250 mL) solution of 6-methyl-7-hydroxycoumarin (9.3 g, 52.8 mmol) was reacted with allyl bromide (10 mL, 118 mmol) in the presence of K<sub>2</sub>CO<sub>3</sub> (10 g) by refluxing the mixture for 5 h. After the mixture was chilled, the K<sub>2</sub>CO<sub>3</sub> was filtered off and washed with fresh Me<sub>2</sub>CO. The filtrate and acetonic washings were concentrated to dryness, and the residue was crystallized from MeOH, giving 6-methyl-7-(allyloxy)coumarin (1a) (8.23 g, 72%): mp 124–125 °C; <sup>1</sup>H NMR  $\delta$  7.59 (1 H, d,  $J$  = 9.5, 4-H), 7.20 (1 H, s, 5-H), 6.74 (1 H, s, 8-H), 6.24–5.82 (1 H, m, 2'-H), 6.22 (1 H, d,  $J$  = 9.5, 3-H), 5.57–5.23 (2 H, m, 3'-H), 4.60 (2 H, ddd,  $J$  = 4.9,  $J$  = 1.5,  $J$  = 1.5, 1'-H), 2.26 (3 H, s, 6-Me).

In the same manner the following 7-O-allyl ethers were prepared from the proper 7-hydroxycoumarins. (i) 4,6-Dimethyl-7-(allyloxy)coumarin (1b): mp 134 °C (MeOH, 87%); <sup>1</sup>H NMR  $\delta$  7.32 (1 H, s, 5-H), 6.74 (1 H, s, 8-H), 6.24–5.84 (1 H, m, 2'-H), 6.10 (1 H, q,  $J$  = 1.2, 3-H), 5.54–5.22 (2 H, m, 3'-H), 4.60 (2 H, ddd;  $J$  = 4.9,  $J$  = 1.5,  $J$  = 1.5, 1'-H), 2.38 (3 H, d,  $J$  = 1.2, 4-Me), 2.28 (3 H, s, 6-Me).

(ii) 5,6-Dimethyl-7-(allyloxy)coumarin (1c): mp 123 °C (MeOH, 76%); <sup>1</sup>H NMR  $\delta$  7.90 (1 H, d,  $J$  = 9.7, 4-H), 6.65 (1 H, s, 8-H), 6.40–5.83 (1 H, m, 2'-H), 6.23 (1 H, d,  $J$  = 9.7, 3-H), 5.54–5.20 (2 H, m, 3'-H), 4.58 (2 H, ddd,  $J$  = 4.9,  $J$  = 1.5,  $J$  = 1.5, 1'-H), 2.40 (3 H, s, 5-Me), 2.21 (3 H, s, 6-Me).

**Claisen Rearrangement.** A solution of 6-methyl-7-(allyloxy)coumarin (1a) (11.43 g; 52.8 mmol) in *N,N*-diethylaniline (100 mL) was refluxed for 2 h. After this time the reaction mixture monitored by TLC showed a single spot, while the starting product was shown to be completely absent. After cooling, *n*-hexane (500 mL) was added to the mixture, and the precipitate obtained was collected, washed several times with *n*-hexane, and crystallized from EtOAc, giving 6-methyl-7-hydroxy-8-allylcoumarin (2a) (6.32 g, 55.3%): mp 161–162 °C; <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>CO)  $\delta$  8.32 (1 H, br s, 7-OH), 7.89 (1 H, d,  $J$  = 9.4, 4-H), 7.37 (1 H, br s, 5-H), 6.35–5.81 (1 H, m, 2'-H), 6.26 (1 H, d,  $J$  = 9.4, 3-H), 5.28–4.97 (2 H, m, 3'-H), 3.72 (2 H, ddd,  $J$  = 6.0,  $J$  = 1.5,  $J$  = 1.5, 1'-H), 2.41 (3 H, d,  $J$  = 0.8, 6-Me).

The following 8-allyl derivatives were obtained analogously from the proper 7-(allyloxy)coumarin. (i) 4,6-Dimethyl-7-hydroxy-8-allylcoumarin (2b): mp 179–180 °C (EtOAc/*n*-hexane, 63%); <sup>1</sup>H NMR  $\delta$  7.27 (1 H, s, 5-H), 6.20–5.76 (1 H, m, 2'-H), 6.12 (1 H, q,  $J$  = 1.2, 3-H), 6.02 (1 H, s, 7-OH), 5.31–5.06 (2 H, m, 3'-H), 3.68 (2 H, ddd,  $J$  = 6.0,  $J$  = 1.5,  $J$  = 1.5, 1'-H), 2.39 (3 H, d,  $J$  = 1.2, 4-Me), 2.31 (3 H, s, 6-Me).

(ii) 5,6-Dimethyl-7-hydroxy-8-allylcoumarin (2c): mp 169–170 °C (EtOAc/cyclohexane, 65%); <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>CO)  $\delta$  8.22 (1 H, d,  $J$  = 9.7, 4-H), 6.26–5.82 (1 H, m, 2'-H), 6.25 (1 H, d,  $J$  = 9.7, 3-H), 5.22–4.94 (2 H, m, 3'-H), 3.72 (2 H, ddd,  $J$  = 6.1,  $J$  = 1.5,  $J$  = 1.5, 1'-H), 2.53 (3 H, s, 5-Me), 2.37 (3 H, s, 6-Me).

**5'-Methylangelicins. (a) 7-Acetoxy-8-allylcoumarins.** A mixture of 6-methyl-7-hydroxy-8-allylcoumarin (2a) (6.31 g, 29 mmol), acetic anhydride (100 mL), and anhydrous AcONa (3 g) was refluxed for 1 h. The reaction mixture was cautiously diluted with H<sub>2</sub>O (100 mL), refluxed for 10 min, and poured into H<sub>2</sub>O (1000 mL). The precipitate was collected, washed with abundant H<sub>2</sub>O, and crystallized from MeOH, giving 6-methyl-7-acetoxy-8-allylcoumarin (3a) (7.0 g, 93%): mp 110–111 °C; <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>CO)  $\delta$  8.02 (1 H, d,  $J$  = 9.6, 4-H), 7.56 (1 H, br s, 5-H), 6.48 (1 H, d,  $J$  = 9.6, 3-H), 6.27–5.77 (1 H, m, 2'-H), 5.32–4.97 (2 H, m, 3'-H), 3.60 (2 H, ddd,  $J$  = 6.1,  $J$  = 1.5,  $J$  = 1.5, 1'-H), 2.50 (3 H, s, 7-OAc), 2.30 (3 H, d,  $J$  = 0.7, 6-Me).

The following 7-acetoxy-8-allylcoumarins were prepared analogously from compounds 2b and 2c. (i) 4,6-Dimethyl-7-acetoxy-8-allylcoumarin (3b): mp 139–140 °C (MeOH, 82%); <sup>1</sup>H NMR  $\delta$  7.34 (1 H, s, 5-H), 6.25 (1 H, q,  $J$  = 1.2, 3-H), 6.15–5.63 (1 H, m, 2'-H), 5.20–4.88 (2 H, m, 3'-H), 3.52 (2 H, ddd,  $J$  = 6.2,  $J$  = 1.5,  $J$  = 1.5, 1'-H), 2.41 (3 H, d,  $J$  = 1.4, 4-Me), 2.36 (3 H, s, 7-OAc), 2.21 (3 H, s, 6-Me).

(ii) 5,6-Dimethyl-7-acetoxy-8-allylcoumarin (3c): mp 148–149 °C (MeOH, 80%); <sup>1</sup>H NMR  $\delta$  7.95 (1 H, d,  $J$  = 9.8, 4-H), 6.37 (1 H, d,  $J$  = 9.8, 3-H), 6.14–5.64 (1 H, m, 2'-H), 5.21–4.89 (2 H, m, 3'-H), 3.50 (2 H, ddd,  $J$  = 6.2,  $J$  = 1.5,  $J$  = 1.5, 1'-H), 2.42 (3 H, s, 5-Me), 2.36 (3 H, s, 7-OAc),  $\delta$  2.11 (3 H, s, 6-Me).

**(b) 8-(2',3'-Dibromopropyl)coumarins.** An acetic solution containing the stoichiometric amount of bromine was dropped at room temperature during a 30-min period into an acetic solution

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(150 mL) of 6-methyl-7-acetoxy-8-allylcoumarin (**3a**) (8.8 g, 34.1 mmol). After the addition was terminated, the solution was stirred for a further 30 min, the solvent was evaporated to dryness, and the residue was crystallized from MeOH, giving 6-methyl-7-acetoxy-8-(2',3'-dibromopropyl)coumarin (**4a**) (11.47 g, 80.5%): mp 152–153 °C;  $^1\text{H NMR}$  ( $(\text{CD}_3)_2\text{CO}$ )  $\delta$  8.04 (1 H, d,  $J = 9.6$ , 4-H), 7.64 (1 H, s, 5-H), 6.50 (1 H, d,  $J = 9.6$ , 3-H), 4.92–4.59 (1 H, m, 2'-H), 4.24–3.30 (4 H, m, 1'-H and 3'-H), 2.56 (3 H, s, 7-OAc), 2.32 (3 H, s, 6-Me).

In a similar manner, the following 8-dibromopropylcoumarins were obtained from compounds **3b** and **3c**. (i) 4,6-Dimethyl-7-acetoxy-8-(2',3'-dibromopropyl)coumarin (**4b**): mp 135–137 °C (MeOH, 74%);  $^1\text{H NMR}$   $\delta$  7.42 (1 H, s, 5-H), 6.25 (1 H, q,  $J = 1.2$ , 3-H), 4.70–4.30 (1 H, m, 2'-H), 4.04–3.14 (4 H, m, 1'-H and 3'-H), 2.42 (3 H, s, 7-OAc), 2.41 (3 H, d,  $J = 1.2$ , 4-Me), 2.23 (3 H, s, 6-Me).

(ii) 5,6-Dimethyl-7-acetoxy-8-(2',3'-dibromopropyl)coumarin (**4c**): mp 168–169 °C (MeOH, 76%);  $^1\text{H NMR}$   $\delta$  7.97 (1 H, d,  $J = 9.8$ , 4-H), 6.40 (1 H, d,  $J = 9.8$ , 3-H), 4.69–4.31 (1 H, m, 2'-H), 4.05–3.18 (4 H, m, 1'-H and 3'-H), 2.44 (6 H, s, 7-OAc and 5-Me), 2.13 (3 H, s, 6-Me).

(c) **Cyclization.** To an ethanolic solution (250 mL) of 6-methyl-7-acetoxy-8-(2',3'-dibromopropyl)coumarin (**4a**) (11.5 g, 27.5 mmol), was added a volume of ethanolic 4% KOH solution until a molar ratio (coumarin/KOH) of 1/10 was reached. The mixture was refluxed for 1.5 h in the dark, chilled, diluted with twice its volume of  $\text{H}_2\text{O}$ , and acidified with 10% HCl. The crude precipitate was collected and chromatographed by  $\text{SiO}_2$  column by eluting with  $\text{CHCl}_3$ . From the pooled first fractions the solvent was evaporated and the residue was crystallized from MeOH, giving 6,5'-dimethylangelicin (**5a**) (2.7 g, 46%): mp 175–176 °C;  $^1\text{H NMR}$   $\delta$  7.68 (1 H, d,  $J = 9.5$ , 4-H), 7.00 (1 H, q,  $J = 0.8$ , 5-H), 6.64 (1 H, q,  $J = 1.1$ , 4'-H), 6.29 (1 H, d,  $J = 9.5$ , 3-H), 2.49 (3 H, d,  $J = 1.1$ , 5'-Me), 2.48 (3 H, d,  $J = 0.8$ , 6-Me).

In a similar manner, the following 5'-methylangelicins were prepared from compounds **4b** and **4c**. (i) 4,6,5'-Trimethylangelicin (**5b**) (MeOH, 39%): mp 183 °C;  $^1\text{H NMR}$   $\delta$  7.17 (1 H, q,  $J = 1.0$ , 5-H), 6.69 (1 H, q,  $J = 1.1$ , 4'-H), 6.20 (1 H, q,  $J = 1.2$ , 3-H), 2.52 (3 H, d,  $J = 1.0$ , 6-Me), 2.50 (3 H, d,  $J = 1.1$ , 5'-Me), 2.45 (3 H, d,  $J = 1.2$ , 4-Me).

(ii) 5,6,5'-Trimethylangelicin (**5c**) (MeOH, 42%): mp 204–205 °C;  $^1\text{H NMR}$   $\delta$  7.99 (1 H, d,  $J = 9.9$ , 4-H), 6.63 (1 H, q,  $J = 1.1$ , 4'-H), 6.31 (1 H, d,  $J = 9.9$ , 3-H), 2.47 (3 H, d,  $J = 1.1$ , 5'-Me), 2.45 and 2.43 (3 H each, s, 5-Me and 6-Me).

**Angelicins without Methyl Groups in the Furan Ring.** Into an EtOAc (200 mL) solution of 6-methyl-7-hydroxy-8-allylcoumarin (**2a**) (2.4 g, 11.1 mmol), cooled in an ice bath, was bubbled a current of ozonized oxygen until 1.1 times the stoichiometric amount had been added. The solution was then submitted immediately to hydrogenation in the presence of Pd 10% on  $\text{CaCO}_3$  (0.3 g), and the mixture was stirred until the rapid absorption of hydrogen ceased. The catalyst was removed by filtration and the solvent was evaporated; to the residue was added 85%  $\text{H}_3\text{PO}_4$  (60 mL) and the mixture was heated at 100 °C for 30 min. The mixture was chilled, diluted with two volumes of water, and extracted with EtOAc. The solvent was evaporated from the dried ( $\text{Na}_2\text{SO}_4$ ) organic phase, and the residue chromatographed on a  $\text{SiO}_2$  column eluting with  $\text{CHCl}_3$ , giving the 6-methylangelicin **6a** crystallized from MeOH (0.58 g, 26%): mp 164–165 °C;  $^1\text{H NMR}$   $\delta$  7.73 (1 H, d,  $J = 9.5$ , 4-H), 7.67 (1 H, d,  $J = 2.2$ , 5'-H), 7.11 (1 H, br s, 5-H), 7.08 (1 H, d,  $J = 2.2$ , 4'-H), 6.33 (1 H, d,  $J = 9.5$ , 3-H), 2.52 (3 H, d,  $J = 0.8$ , 6-Me).

The following angelicins were obtained analogously from compounds **2b** and **2c**. (i) 4,6-Dimethylangelicin (**6b**) (EtOAc/cyclohexane, 22%): mp 150–151 °C;  $^1\text{H NMR}$   $\delta$  7.67 (1 H, d,  $J = 2.2$ , 5'-H), 7.28 (1 H, br s, 5-H), 7.13 (1 H, d,  $J = 2.2$ , 4'-H), 6.24 (1 H, q,  $J = 1.2$ , 3-H), 2.57 (3 H, d,  $J = 0.9$ , 6-Me), 2.48 (3 H, d,  $J = 1.2$ , 4-Me).

(ii) 5,6-Dimethylangelicin (**6c**) (MeOH, 23%): mp 219–220 °C;  $^1\text{H NMR}$   $\delta$  8.01 (1 H, d,  $J = 9.9$ , 4-H), 7.61 (1 H, d,  $J = 2.2$ , 5'-H), 7.05 (1 H, d,  $J = 2.2$ , 4'-H), 6.34 (1 H, d,  $J = 9.9$ , 3-H), 2.47 (6 H, s, 5-Me and 6-Me).

**4-Methylangelicins. (a) 7-Acetoxycoumarins.** In the usual way the 6-methyl-, 4,6-dimethyl-, and 5,6-dimethylumbelliferones were acetylated, obtaining the following 7-acetoxycoumarins. (i) 6-Methyl-7-acetoxycoumarin (**7a**): mp 148–149 °C (MeOH) [lit.<sup>14</sup>

mp 147 °C];  $^1\text{H NMR}$   $\delta$  7.63 (1 H, d,  $J = 9.5$ , 4-H), 7.32 (1 H, br s, 5-H), 7.05 (1 H, s, 8-H), 6.36 (1 H, d,  $J = 9.5$ , 3-H), 2.36 (3 H, s, 7-OAc), 2.22 (3 H, br s, 6-Me).

(ii) 4,6-Dimethyl-7-acetoxycoumarin (**7b**): mp 160–161 °C (MeOH);  $^1\text{H NMR}$   $\delta$  7.43 (1 H, br s, 5-H), 7.04 (1 H, s, 8-H), 6.24 (1 H, q,  $J = 1.2$ , 3-H), 2.41 (3 H, d,  $J = 1.2$ , 4-Me), 2.35 (3 H, s, 7-OAc), 2.24 (3 H, br s, 6-Me).

(iii) 5,6-Dimethyl-7-acetoxycoumarin (**7c**): mp 206 °C (MeOH);  $^1\text{H NMR}$   $\delta$  7.94 (1 H, d,  $J = 9.9$ , 4-H), 6.92 (1 H, s, 8-H), 6.38 (1 H,  $J = 9.9$ , 3-H), 2.44 (3 H, s, 5-Me), 2.36 (3 H, s, 7-OAc), 2.14 (3 H, s, 6-Me).

(b) **Fries Rearrangement.** An accurately mixed mixture of 6-methyl-7-acetoxycoumarin (**7a**) (4.0 g, 18.3 mmol), anhydrous aluminum chloride (8.0 g, 60 mmol), and anhydrous sodium chloride (4.0 g, 68 mmol) was heated at 170 °C for 1.5 h. The cooled mixture was added to dilute HCl (50 mL), refluxed for 10 min, diluted with water (200 mL), and extracted three times with EtOAc (100 mL). From the dried ( $\text{Na}_2\text{SO}_4$ ) organic phase the solvent was evaporated and the residue was crystallized two times from MeOH, giving 6-methyl-7-hydroxy-8-acetylcoumarin (**8a**) (1.80 g, 45%): mp 170 °C;  $^1\text{H NMR}$   $\delta$  13.22 (1 H, s, 7-OH), 8.16 (1 H, d,  $J = 9.7$ , 4-H), 7.67 (1 H, q,  $J = 0.8$ , 5-H), 6.36 (1 H, d,  $J = 9.7$ , 3-H), 2.64 (3 H, s, 8-Ac), 2.37 (3 H, d,  $J = 0.8$ , 6-Me).

In a similar manner, the following 8-acetylcoumarins were prepared from compounds **7b** and **7c**. (i) 4,6-Dimethyl-7-hydroxy-8-acetylcoumarin (**8b**) (EtOAc, 48%): mp 215–216 °C;  $^1\text{H NMR}$   $\delta$  13.94 (1 H, s, 7-OH), 7.51 (1 H, q,  $J = 0.8$ , 5-H), 6.14 (1 H, q,  $J = 1.2$ , 3-H), 2.96 (3 H, s, 8-Ac), 2.41 (3 H, d,  $J = 1.2$ , 4-Me), 2.28 (3 H, d,  $J = 0.8$ , 6-Me).

(ii) 5,6-Dimethyl-7-hydroxy-8-acetylcoumarin (**8c**) (EtOAc; 60%): mp 236–237 °C;  $^1\text{H NMR}$   $\delta$  14.23 (1 H, s, 7-OH), 7.94 (1 H, d,  $J = 9.8$ , 4-H), 6.27 (1 H, d,  $J = 9.8$ , 3-H), 2.94 (3 H, s, 8-Ac), 2.45 (3 H, s, 5-Me), 2.23 (3 H, s, 6-Me).

(c) **Ethyl (7-Coumarinyloxy)acetates.** An acetone solution (100 mL) of 6-methyl-7-hydroxy-8-acetylcoumarin (**8a**) (1.8 g, 8.2 mmol), ethyl bromoacetate (3 mL, 27 mmol), and anhydrous  $\text{K}_2\text{CO}_3$  (3.0 g) was refluxed 4 h. From the chilled mixture the solid was filtered off and washed with fresh acetone, and the solvent was evaporated. The residue was chromatographed on a  $\text{SiO}_2$  column by eluting with  $\text{CHCl}_3$ . From the first pooled fractions the solvent was eliminated and the residue was crystallized from MeOH, giving ethyl [(6-methyl-8-acetyl-7-coumarinyloxy)acetate (**9a**) (1.88 g, 75%): mp 88–89 °C;  $^1\text{H NMR}$   $\delta$  7.63 (1 H, d,  $J = 9.6$ , 4-H), 7.34 (1 H, q,  $J = 0.8$ , 5-H), 6.34 (1 H, d,  $J = 9.6$ , 3-H), 4.52 (2 H, s,  $\text{OCH}_2$ ), 4.27 (2 H, q,  $J = 7.1$ ,  $\text{CH}_2\text{CH}_3$ ), 2.67 (3 H, s, 8-Ac), 2.35 (3 H, d,  $J = 0.8$ , 6-Me), 1.31 (3 H, t,  $J = 7.1$ ,  $\text{CH}_2\text{CH}_3$ ).

The following ethyl (7-coumarinyloxy)acetates were obtained analogously from compounds **8b** and **8c**. (i) Ethyl [(4,6-dimethyl-8-acetyl-7-coumarinyloxy)acetate (**9b**) (MeOH, 70%): mp 96–97 °C;  $^1\text{H NMR}$   $\delta$  7.46 (1 H, q,  $J = 0.8$ , 5-H), 6.21 (1 H, q,  $J = 1.3$ , 3-H), 4.51 (2 H, s,  $\text{OCH}_2$ ), 4.27 (2 H, q,  $J = 7.1$ ,  $\text{CH}_2\text{CH}_3$ ), 2.65 (3 H, s, 8-Ac), 2.41 (3 H, d,  $J = 1.3$ , 4-Me), 2.37 (3 H, d,  $J = 0.8$ , 6-Me), 1.32 (3 H, t,  $J = 7.1$ ,  $\text{CH}_2\text{CH}_3$ ).

(ii) Ethyl [(5,6-dimethyl-8-acetyl-7-coumarinyloxy)acetate (**9c**) (EtOAc/*n*-hexane, 63%): mp 109 °C;  $^1\text{H NMR}$   $\delta$  7.93 (1 H, d,  $J = 9.8$ , 4-H), 6.36 (1 H, d,  $J = 9.8$ , 3-H), 4.48 (2 H, s,  $\text{OCH}_2$ ), 4.28 (2 H, q,  $J = 7.2$ ,  $\text{CH}_2\text{CH}_3$ ), 2.65 (3 H, s, 8-Ac), 2.44 (3 H, s, 5-Me), 2.28 (3 H, s, 6-Me), 1.31 (3 H, t,  $J = 7.2$ ,  $\text{CH}_2\text{CH}_3$ ).

(d) **(7-Coumarinyloxy)acetic Acids.** A 5% KOH aqueous methanolic (1:1) (100 mL) solution of ethyl [(6-methyl-8-acetyl-7-coumarinyloxy)acetate (**9a**) (3.0 g, 9.9 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 and washed several times with water. The mother liquors were extracted three times with EtOAc (100 mL), and the solvent was evaporated from the dried ( $\text{Na}_2\text{SO}_4$ ) organic phase, obtaining a further crop of product. The pooled crude products were crystallized from EtOAc/*n*-hexane, giving [(6-methyl-8-acetyl-7-coumarinyloxy)acetic acid (**10a**) (1.83 g, 66.8%): mp 196–197 °C;  $^1\text{H NMR}$  ( $(\text{CD}_3)_2\text{CO}$ )  $\delta$  8.03 (1 H, d,  $J = 9.7$ , 4-H), 7.70 (1 H, q,  $J = 0.8$ , 5-H), 6.47 (1 H, d,  $J = 9.7$ , 3-H), 4.71 (2 H, s,  $\text{OCH}_2$ ), 2.73 (3 H, s, 8-Ac), 2.48 (3 H, d,  $J = 0.8$ , 6-Me).

In a similar manner, the following (7-coumarinyloxy)acetic acids were prepared from the ethyl esters **9b** and **9c**. (i) [(4,6-Dimethyl-8-acetyl-7-coumarinyloxy)acetic acid (**10b**) (EtOAc, 62%): mp 184–186 °C;  $^1\text{H NMR}$  ( $(\text{CD}_3)_2\text{CO}$ )  $\delta$  7.81 (1 H, q,  $J = 0.8$ , 5-H),

6.36 (1 H, q,  $J = 1.3$ , 3-H), 5.57 (1 H, very br s, COOH), 4.70 (2 H, s, OCH<sub>3</sub>), 2.72 (3 H, s, 8-Ac), 2.57 (3 H, d,  $J = 1.3$ , 4-Me), 2.50 (3 H, d,  $J = 0.8$ , 6-Me).

(ii) [(5,6-Dimethyl-8-acetyl-7-coumarinyl)oxy]acetic acid (**10c**) (MeOH, 65.9%): mp 219–220 °C; <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>CO) δ 8.34 (1 H, d,  $J = 10.0$ , 4-H), 6.47 (1 H, d,  $J = 10.0$ , 3-H), 4.65 (2 H, s, OCH<sub>3</sub>), 2.72 (3 H, s, 8-Ac), 2.62 (3 H, s, 5-Me), 2.44 (3 H, s, 6-Me).

(e) **Cyclization**. A mixture of [(6-methyl-8-acetyl-7-coumarinyl)oxy]acetic acid (**10a**) (3.7 g, 13.4 mmol), Ac<sub>2</sub>O (70 mL), and anhydrous sodium acetate (4.0 g) was refluxed for 1 h; chilled mixture water (100 mL) was cautiously added and the mixture refluxed 10 min, diluted with water (200 mL), and extracted three times with EtOAc (200 mL). The organic phase was washed three times with saturated NaHCO<sub>3</sub> solution (100 mL) and dried, and the solvent was evaporated. The residue was crystallized from MeOH, giving 6,4'-dimethylangelicin (**11a**) (2.1 g, 73%): mp 157–158 °C; <sup>1</sup>H NMR δ 7.72 (1 H, d,  $J = 9.6$ , 4-H), 7.42 (1 H, q,  $J = 1.3$ , 5'-H), 7.10 (1 H, q,  $J = 0.8$ , 5-H), 6.33 (1 H, d,  $J = 9.6$ , 3-H), 2.51 (3 H, d,  $J = 1.3$ , 4'-Me), 2.49 (3 H, d,  $J = 0.8$ , 6-Me).

The pooled alkaline washings were acidified by dilute HCl and extracted many times with EtOAc. The solvent was evaporated from the dried (Na<sub>2</sub>SO<sub>4</sub>) organic phase, obtaining a scarce residue (0.2 g, 5.7%) easily soluble in NaHCO<sub>3</sub> solution, mp 315 °C dec, which was considered to be the undecarboxylated intermediate product **12a**.

The following 4'-methylangelicins were obtained analogously from the acids **10b** and **10c**. (i) 4,6,4'-Trimethylangelicin (**11b**) (MeOH, 64%): mp 201–202 °C; <sup>1</sup>H NMR δ 7.42 (1 H, q,  $J = 1.3$ , 5'-H), 7.25 (1 H, br s, 5-H), 6.21 (1 H, q,  $J = 1.2$ , 3-H), 2.52 (3 H, d,  $J = 0.8$ , 6-Me), 2.51 (3 H, d,  $J = 1.3$ , 4'-Me), 2.46 (3 H, d,  $J = 1.2$ , 4-Me).

(ii) 5,6,4'-Trimethylangelicin (**11c**) (MeOH, 70%): mp 229–230 °C; <sup>1</sup>H NMR δ 8.02 (1 H, d,  $J = 9.9$ , 4-H), 7.35 (1 H, q,  $J = 1.3$ , 5'-H), 6.33 (1 H, d,  $J = 9.9$ , 3-H), 2.49 (3 H, d,  $J = 1.3$ , 4'-Me), 2.47 and 2.44 (3 H each, br s, 5-Me and/or 6-Me).

**DNA**. Calf thymus DNA (cat. D 1501) was purchased from Sigma Chemical Co., St. Louis, MO. Hypochromicity of the sample, determined according to Marmur and Doty,<sup>25</sup> 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.) were used; in one part of the cell the aqueous solution of a labeled furocoumarin<sup>3</sup> 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  $3 \times 10^{-3}$  and  $3 \times 10^{-4}$  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  $25 \pm 0.05$  °C in the dark.<sup>3,5</sup>

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.<sup>26</sup>

In order to evaluate the extent of furocoumarin complexed to DNA (100% of reactant at 0 time) for calculation of the rate constants of the photoreactions,<sup>3,5</sup> analogous experiments were made, however at lower ionic strength (2 mmol of NaCl); moreover, the concentration of the furocoumarins and of 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%. The compounds have been labeled according to ref 10; they had the following specific activity (Ci/mol): **1** = 240, **5a** = 8.69, **5b** = 10.2, **5c** = 10.5, **6a** = 13.1, **6b** = 7.70, **11a** = 9.50, **11b** = 10.6.

**Computation of the Interaction Parameters**. The method of computation involved an iterative procedure capable of satisfying the following equation of McGhee and von Hippel:<sup>15</sup>

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

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 calculated binding isotherm at 5% saturation increments.

**Irradiation Procedure**. Aqueous solutions ( $2.3 \times 10^{-3}$  M) of DNA containing  $2 \times 10^{-3}$  M NaCl and  $1 \times 10^{-3}$  M EDTA were added to the labeled furocoumarin to be examined ( $3 \times 10^{-5}$  M). Measured volumes (2 mL) of the prepared solutions were introduced into calibrated glass tubes, and these were immersed in 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);<sup>27</sup> the irradiation intensity determined by using a chemical actinometer<sup>28</sup> was  $1.07 \times 10^{16}$  quanta s<sup>-1</sup> mL<sup>-1</sup>. After irradiation, the macromolecule was precipitated by addition of NaCl up to 2 M and 2 volumes of absolute ethanol, washed with 80% ethanol, and redissolved in the initial volume of water. The solutions so obtained were used for radiochemical measurements.<sup>3</sup>

**Evaluation of Cross-Linkages**. This evaluation was made directly on the irradiated DNA samples, without precipitation, according to ref 3 and 5.

**DNA Synthesis in Ehrlich Cells**. Ehrlich ascites tumor cells in Hanks' solution (aliquots of 5 mL;  $2 \times 10^7$  cell/mL) were irradiated in Petri dishes (5-cm diameter) placed on ice by a Philips HPW 125 set at 20-cm distance ( $2.02 \times 10^{16}$  quanta/s incident on the whole sample). Samples of  $2 \times 10^6$  of the irradiated cells in 0.5 mL in the same medium containing 1 μCi of [<sup>3</sup>H]-thymidine were incubated for 15 min at 37 °C. The reaction was stopped by chilling in ice and adding 1 mL of 5 mM of the unlabeled nucleoside. The cells were collected by filtering on Whatman CF/c dishes (diameter 2.5 cm), then washed three times with saline, and treated with 10 mL 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 acid and expressed as percentage of the incorporation observed in the untreated cells (2000 DPM/μg for reference DNA). DNA content was determined by the diphenylamine<sup>29</sup> reaction. The data were submitted to the probit analysis and expressed as the ID<sub>50</sub>, i.e., the UV-A radiation dose that in the presence of the  $1.9 \times 10^{-5}$  M produces a 50% inhibition.

**Mutagenesis Tests**. Bacterial strains: *Escherichia coli* B strain WP2 uvr A was obtained from Dr. Verritt; this strain carries a nonsense mutation in the trp E gene which is reverted by UV light and by most base pair substitution mutagens.<sup>21</sup> *Salmonella typhimurium* TA 98 and TA 100 strains were obtained from Dr. B. N. Ames and show the following properties: TA 98 his D 35052 (frameshift mutation), uvr B rfa/p KM 101; TA 100 his G 46 (missense mutation) uvr B rfa/p KM 101.<sup>30</sup>

For mutagenic test by UV-A irradiation, *E. coli* cells were suspended in phosphate-buffered saline ( $4 \times 10^8$  cells/mL), containing the furocoumarin to be studied (20 μM), and after incubation for 20 min in the dark, they were irradiated with a

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"Blacklite" blue fluorescent lamp (FT15T8BLB, 15 W), placed at 30-cm distance.

Revertant colonies were determined on agar prepared with semienriched medium (SEM) agar (minimum medium agar (MMA) fortified with 0.1 mg/mL of Difco nutrient broth) after 48 h of incubation at 37 °C.

For dark mutagenesis, various amounts of compounds were added to molten top agar (containing 0.5 mM L-histidine and 0.5 mM biotine, 0.1 mL of an overnight culture of *Salmonella typhimurium* TA 98) and then poured onto plates prepared with Vogel-Bonner agar.<sup>30</sup> Plates were incubated in the dark at 37 °C for 48 h.

For dark mutagenesis determined after metabolic activation, the *Salmonella typhimurium* TA 100 strain was used. The metabolic activation was carried out by means of S-9 Mix,<sup>21</sup> and

the angelicins were tested in a concentration range between 5 and 20 µg/mL.

**Acknowledgment.** We thank M. Peron for her skillful technical assistance. Part of the research was carried out with the financial support of the Ministry of Public Instruction (Rome) (40% funds).

**Registry No.** 1a, 90369-99-6; 1b, 90370-00-6; 1c, 90370-01-7; 2a, 90370-02-8; 2b, 90370-03-9; 2c, 90370-04-0; 3a, 90370-05-1; 3b, 90370-06-2; 3c, 90370-07-3; 4a, 90370-08-4; 4b, 90370-09-5; 4c, 90370-10-8; 5a, 90370-11-9; 5b, 90370-12-0; 5c, 90370-13-1; 6a, 90370-14-2; 6b, 90370-15-3; 6c, 90370-16-4; 7a, 53811-55-5; 7b, 90370-17-5; 7c, 90370-18-6; 8a, 90370-19-7; 8b, 90370-20-0; 8c, 90370-21-1; 9a, 90370-22-2; 9b, 90370-23-3; 9c, 90370-24-4; 10a, 90370-25-5; 10b, 90370-26-6; 10c, 90370-27-7; 11a, 90370-28-8; 11b, 90370-29-9; 11c, 90370-30-2; 6-methyl-7-hydroxycoumarin, 53811-57-7; 4,6-dimethyl-7-hydroxycoumarin, 1484-98-6; 5,6-dimethyl-7-hydroxycoumarin, 90370-31-3; ethyl bromoacetate, 105-36-2.

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## Design, Synthesis, and Testing of Potential Antisickling Agents. 4. Structure-Activity Relationships of Benzyloxy and Phenoxy Acids

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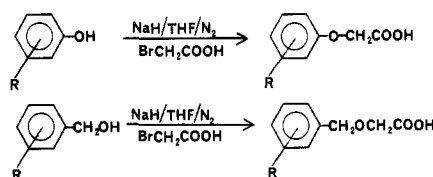
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Received January 26, 1984

In this paper we further establish the activity of two classes of small molecules, benzyloxy and phenoxy acids, as potent inhibitors of hemoglobin S (HbS) gelation. Structural modifications with a large number of each class confirm our earlier work that the highest activity is observed with compounds that contain dihalogenated aromatic rings with attached polar side chains. We have also found a halogenated aromatic malonic acid derivative to be quite active. Compounds reported in this paper are compared with other antigelling agents studied in our laboratory. Comments are made concerning the antigelling activity and binding sites of four derivatives and their effect on the allosteric mechanism of hemoglobin (Hb) function.

Several groups have investigated the effects of aromatic compounds as antigelling agents.<sup>1-5</sup> During the last several years we also initiated a program to design, synthesize, and test small molecules that might be suitable as therapeutic agents in the treatment of sickle cell anemia. In our search for active agents we have employed three different methods of design and all have produced active compounds. One approach involved the design of agents modeled to bind stereospecifically to the surface of hemoglobin (Hb) at or near important contact areas in the hemoglobin S (HbS) polymer as indicated from the X-ray crystal and fiber structures of HbS. This approach produced the very active meta-disubstituted benzoic acids.<sup>6</sup> A second approach that was also successful in producing very active molecules involved determination of the binding sites of weakly active antigelling agents with use of X-ray diffraction studies<sup>7</sup> and subsequent redesign of agents to better fit that site using the binding information. The phenoxyacetic acids were discovered by using this approach.<sup>8</sup> The third method involved modification of a

Scheme I



moderately active but insoluble antigelling agent, *p*-bromobenzyl alcohol,<sup>9</sup> by addition of a polar side chain to increase its solubility. This idea produced the very active (benzyloxy)acetic acids.<sup>8</sup> All three methods produced compounds with some overlapping structural features that appear to impart strong antigelling activity. Specifically, it has been shown that a mono- or dihalogenated aromatic acid with a polar side chain contains the appropriate moieties to bind effectively to HbS at sites that destabilize the HbS gel. Significant antigelling activity of other halogenated aromatics similar to ours has also been reported by three groups.<sup>10-12</sup>

This paper expands our earlier work<sup>8</sup> and attempts to rationalize the antigelling activity of the benzyloxy and phenoxy acids with our more recent X-ray studies as well as gives a hypothesis about a mechanism to explain their

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