Novel Pyrone Side Tetracyclic Psoralen Derivatives: Synthesis and Photobiological Evaluation

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This study reports the synthesis of tetrahydrobenzo- (**4**−**6**) and benzopsoralen (**7**−**9**) derivatives obtained by condensing the fourth ring to the pyrone side of the tricyclic psoralen moiety. The new compounds are characterized by having a methoxy, a hydroxy, or a dimethylaminopropoxy side chain inserted at position 8 of the psoralen chromophore. The evaluation of the photoantiproliferative activity on human tumor cell lines along with skin phototoxicity on guinea pigs revealed an interesting photobiological pattern for the dimethylaminopropoxy derivatives **6** and **9**: they are in fact able to exert an antiproliferative effect up to 1 order of magnitude higher than that of the well-known drug 8-MOP, but they are devoid of skin phototoxicity. The ability of both **6** and **9** to photoadd to DNA is demonstrated by the isolation and characterization of the 4′,5′-monoadducts. AM1 calculations were also performed to gain further insight into the molecular basis of their photobiological behavior.

Introduction

Photochemotherapy constitutes an intriguing approach for the treatment of hyperproliferative diseases.[1] PUVA therapy (psoralen plus UVA light) plays a significant role in this field. Its usefulness in the treatment of skin hyperproliferative diseases (such as psoriasis)[2−4] or for the cutaneous T-cell lymphoma[5,6] has been well-established. However, PUVA treatment is limited by both short-term (erythema, hyperpigmentation) and long-term (benign keratoses, premalignant keratoses, skin cancers) side effects. Of the psoralen structures, 8-methoxypsoralen (8-MOP) is the drug most widely employed in PUVA therapy, but also its congener 5-methoxypsoralen (5-MOP) is successfully employed, even thought to a lesser extent.[3,7] Their cellular target(s) and their molecular mechanism of action have been already clarified. In particular, psoralens are able to absorb a photon from UVA light (365 nm) to give rise to a covalent adduct with the pyrimidine bases of DNA, mainly thymine, by means of a photoaddition reaction involving the 4′,5′-furan-side double bond and/or the 3,4-pyrone-side double bond of the tricyclic moiety and the 5,6-double bond of the base. The ability of the furan side monoadduct to photoreact further, causing the 3,4-double bond to form a diadduct, has been demonstrated for psoralens.[8]

With the aim of developing drugs endowed with a better photochemotherapeutic pattern, i.e., high antiproliferative ability along with lower skin photosensitization, a large number of new derivatives have been synthesized and studied.[9−11] In particular, a synthetic approach led to the condensation of a benzenic ring at the photoreactive 4′,5′-double bond, making it possible to obtain new tetracyclic structures that showed a photobinding ability toward DNA comparable to that of 8-MOP, notwithstanding their monofunctional behavior.[10] Moreover, some methylpsoralen and hydroxymethylpsoralen derivatives, also characterized by the presence of a fourth ring (benzenic or cyclohexenylic) fused to the 4′,5′-position, stimulated a further interest in these tetracyclic structures. Indeed, they appeared to be almost devoid of skin phototoxicity and characterized by a better ability to interact with DNA versus 8-MOP.[12] Nevertheless, the biological potentiality of this new moiety appeared to be limited by a low aqueous solubility. In this connection, a subsequent line of research proposed the insertion in the tetracyclic chromophore of a basic side chain protonable at physiological pH. The results obtained by inserting a dimethylaminopropoxy side chain in position 11 or 5, in analogy with the well-known drugs 8-MOP and 5-MOP, appeared very promising.[13,14] In particular, for the new tetracyclic water-soluble structures thus obtained, an effective interaction with DNA along with a remarkable antiproliferative activity was achieved. These characteristics appeared to be more pronounced for the analogues of 8-MOP than for those of 5-MOP. In addition, noteworthy was the decrease of erythema induction for the tetrahydrobenzo derivatives and the lack of any skin photosensitizing effects for the benzo derivatives, in contrast to the parent drugs.[14]

On the basis of such promising results, it appeared to be of interest to pursue research into the tetracyclic psoralen derivatives. In this connection, the condensation of the fourth nucleus (benzenic or cyclohexenylic) at the pyrone side of the psoralen molecule,[15−17] instead of the furan one, can allow further in-depth investigation into the photobiological role of the tetracyclic psoralen moieties.

In this paper the synthesis of six new tetracyclic derivatives, characterized by having the fourth aromatic

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or cyclohexenyl ring condensed at the 3,4-double bond of the pyrone belonging to the psoralen moiety, is reported. Both tetrahydrobenzo- (**4**−**6**) and benzo derivatives (**7**−**9**) carrying a methoxy, a hydroxy, and a dimethylaminopropoxy substituent at position 11 were studied. In particular, the antiproliferative activity on human tumor cell lines, skin phototoxicity, and interaction with DNA were investigated. Finally, the isolation and characterization of the furan photoadduct between both the tetrahydrobenzo- (**6**) and benzodimethylaminopropoxy derivatives (**9**) with thymine are reported.

Furthermore, we have tried to devise a very simple theoretical model of the binding of **6**, **9**, and the related compounds having the fourth ring (cyclohexenylic or benzenic) fused at the furan side to DNA, to gain further possible hints about the photobiological role of these moieties.

Results and Discussion

Chemistry. The compounds studied (**4**−**9**) were obtained starting from 2-methoxyresorcinol (**1**), as shown in Scheme 1. Pechmann condensation by treatment of compound **1** with ethyl 2-oxocyclohexanecarboxylate afforded the corresponding hydroxycoumarin **2**, which through a Williamson reaction with chloroacetone (refluxing acetone/K_2CO_3, 24 h) gave the oxo ether **3** in 71% yield. Cyclization of **3** by heating in strongly alkaline solution afforded the tetrahydrobenzofurocoumarin **4** in 82% yield. The unsubstituted terminal ring of **4** was aromatized by heating with DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) in refluxing toluene, which afforded the benzofurocoumarin **7** in 60% yield.

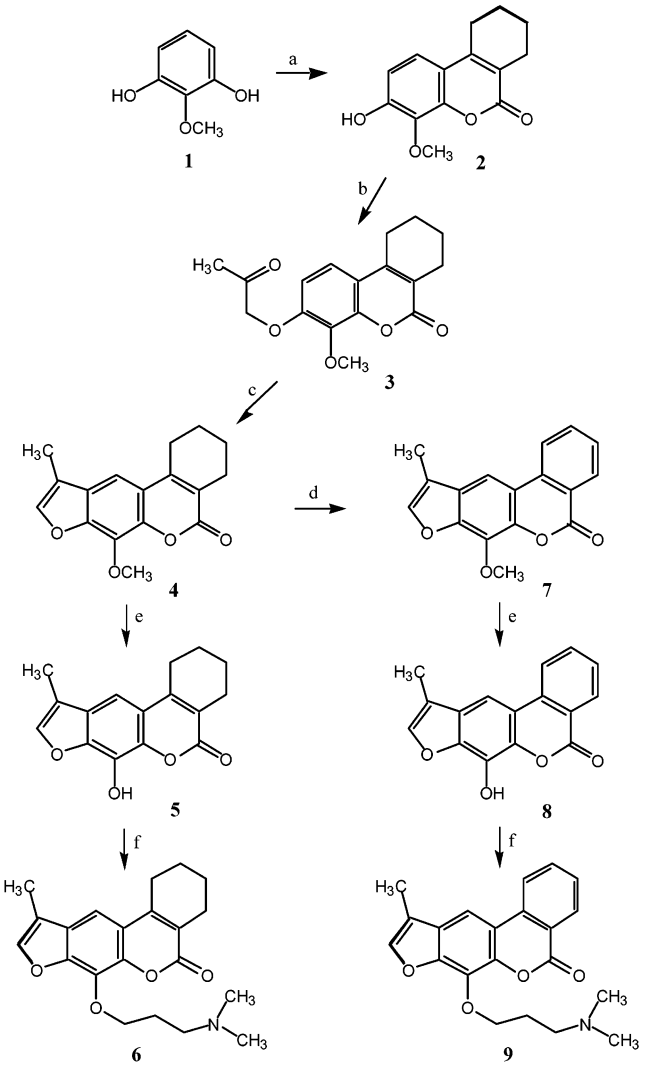
The methoxypsoralen derivatives **4** and **7** were transformed to **5** and **8** in 87% and 78% yields, respectively, by hydrolysis of their methoxy groups to hydroxyl groups with aluminum trichloride in refluxing methylene chloride. Finally, treatment of compounds **5** and **8** with 3-chloro-*N*,*N*-dimethylpropylamine and NaH in the presence of NaI in refluxing dimethylformamide afforded compounds **6** and **9** in 62% and 67% yields, respectively.

Photobiological Activity. The antiproliferative activity of **4**−**9** was evaluated on two human tumor cell lines: HeLa (human cervix adenocarcinoma cells) and HL-60 (human promyelocytic leukemic cells). The well-known photochemotherapeutic drug 8-MOP was tested under the same experimental conditions and considered as reference compound. The results have been expressed as IC_{50} values, i.e., the concentration of compound able to induce the death of 50% of the cells with respect to a control culture. Table 1 reports the data relative to all new tetracyclic compounds and 8-MOP, for both cell lines taken into account.

After exposure to UVA light (0.793 J cm^{-2} at 365 nm) the compounds characterized by having the dimethylaminopropoxy side chain, both tetrahydrobenzo- (**6**) and benzopsoralen (**9**), are able to exert a noticeable antiproliferative ability. In detail, the measured IC_{50} values appear to be from 5 to 15 times lower with respect to those obtained for the reference compound.

As regards the methoxy derivatives (**4** and **7**), they show an antiproliferative activity weaker with respect to that detected for the drug and always significantly lower if compared with that exerted by the dimethylaminopropoxy derivatives, in both the examined cell lines. With respect to the hydroxy derivatives, it can be stated that they are the less active compounds. Particularly, **8** is inactive toward both cell lines, while for **5** a very low level of activity can be detected.

Scheme 1[a]



[a] Reagents: (a) ethyl 2-oxocyclohexanecarboxylate, H_2SO_4; (b) chloroacetone, K_2CO_3, acetone; (c) NaOH; (d) DDQ, toluene; (e) $AlCl_3$, CH_2Cl_2; (f) 3-chloro-*N*,*N*-dimethylpropylamine, NaH, NaI, DMF.

Table 1. Cell Growth Inhibition and Skin Phototoxicity in Guinea Pigs in the Presence of Examined Compounds and 8-MOP as Reference Drug

	IC_{50} (μM) of cell lines				
	HeLa		HL-60		erythema
compd	dark	UVA	dark	UVA	intensity[a]
4	>20	15.7 ± 1.3	>20	13.0 ± 0.8	−
5	>20	17.4 ± 0.2	>20	17 ± 1	−
6	>20	0.68 ± 0.08	16 ± 1	0.95 ± 0.13	−
7	>20	>20	>20	11.8 ± 0.3	−
8	>20	>20	>20	>20	−
9	>20	0.64 ± 0.16	9.9 ± 0.7	0.67 ± 0.11	−
8-MOP	>20	10 ± 3	>20	5.4 ± 0.7	+ (with edema)

[a] +, strong; −, absent.

The absence of cytotoxic effects in the dark, as usually noticed with the psoralen moiety, is also confirmed for the new compounds, except that in the case of the most active, **6** and **9**, in the HL-60 cells. Nevertheless, the measured values are more than 1 order of magnitude higher with the respect to those obtained upon UVA irradiation.

The skin phototoxicity of the new tetracyclic derivatives was determined by evaluating the appearance of erythema, a marker of cutaneous sensitization, on the depilated skin of guinea pigs according to the procedure described in the Experimental Section. The results, reported in Table 1, clearly indicate that all the new derivatives, unlike 8-MOP, are nonphototoxic, even when tested at concentrations higher than that of the reference drug.

With regard to the benzopsoralen derivatives **7**−**9**, the lack of skin phototoxicity does not constitute an unusual effect. Indeed, the same result has already been obtained also for the benzopsoralens characterized by the condensation of the fourth ring at the 4′,5′-double bond and carrying the same chromophoric structure as **7**−**9**, as previously published.[14] In contrast, some interesting features emerge if the two tetracyclic tetrahydrobenzopsoralen moieties, derived from the condensation of the fourth ring at the 4′,5′- (see ref 14) or 3,4-double bond (compound **4**−**6**), are compared. In this case, the condensation at the pyrone side photoreactive double bond appears to be clearly more promising with respect to that at the furan side. Indeed, the dimethylaminopropoxy derivative carrying the cyclohexenyl ring fused at 4′,5′ showed a certain phototoxic ability,[14] while the correspondent compound **6** appears to be devoid of erythematous effect, even though both are able to exert a noticeable and comparable cytotoxic activity on both HeLa and HL-60 cells.

Noncovalent Binding to DNA. The ability of the tricyclic psoralen structure in the ground state to form a complex with DNA via an intercalation mode of binding is well-established. The formation of a molecular complex between the tetracyclic derivatives **6** and **9** and the macromolecule has been investigated by means of flow linear dichroism experiments. The spectra of DNA solutions in the presence of increasing amounts of the considered compounds are reported in Figure 1A (compound **6**) and B (compound **9**).

All dichroic spectra show a strong negative signal at 260 nm, typical of the macromolecule; nevertheless, in the presence of the tested compounds (traces b−d), a further dichroic signal appears at higher wavelengths (300−360 nm). Due to the fact that no contribution from DNA base pairs can be detected in this spectral range, this signal has to be attributed to the added compounds which, on the contrary, absorb in this region. Since small molecules, such as **6** and **9**, cannot themselves become oriented in the flow field, the presence of this signal proves that these compounds have formed a molecular complex with DNA that allows them to undergo an orientation. The negative LD signal of **6** and **9** at wavelengths different from those of DNA is of the same sign as the strong LD band at 260 nm derived from the purine and pyrimidine base pairs. Assuming that also for benzo- and tetrahydrobenzopsoralens, like in psoralens, all strong absorptions are $\pi \rightarrow \pi^*$ transitions

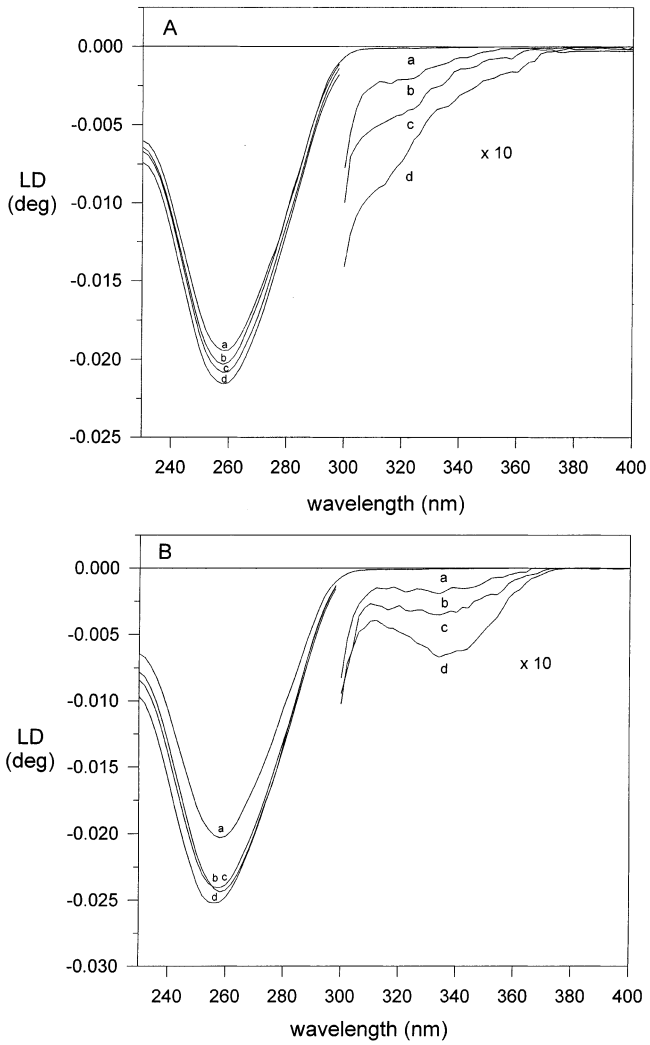


Figure 1. Linear flow dichroism spectra for compounds **6** (A) and **9** (B) at different [drug]/[DNA] ratios: $a = 0$; $b = 0.02$; $c = 0.04$; $d = 0.08$. [DNA] = 1.6×10^{-3} M.

that are polarized in the plane of the chromophore,[18] the noted negative signal must be ascribable to a parallel orientation to the plane of DNA bases. This is in agreement with an intercalative mode of binding.

Flow linear dichroism experiments have been performed also for compounds **4**, **5**, **7**, and **8**. Nevertheless, for these derivatives, no significant dichroic signal appeared at high wavelengths, thus indicating a limited ability to interact with the macromolecule.

Photobinding to DNA. It is well-known that upon UVA irradiation, the 4′,5′-and 3,4-double bonds of the psoralen chromophore are able to give rise to a C_4-cycloaddition involving the 5,6-double bond of pyrimidine bases, mainly thymine. Previous studies have demonstrated that the presence of a fourth ring condensed to the furan side can variously influence its capacity to photoreact. In detail, the condensation of a cyclohexenyl ring did not compromise its ability to form a photoadduct, while in the presence of a benzene ring this capacity appeared completely abolished.[12,14] In particular, the electronic delocalization that takes place in this latter case in the tetracyclic system may be considered responsible for the inability of the involved double bond to photoadd to DNA bases. On the contrary, the steric hindrance caused by the lack of aromaticity

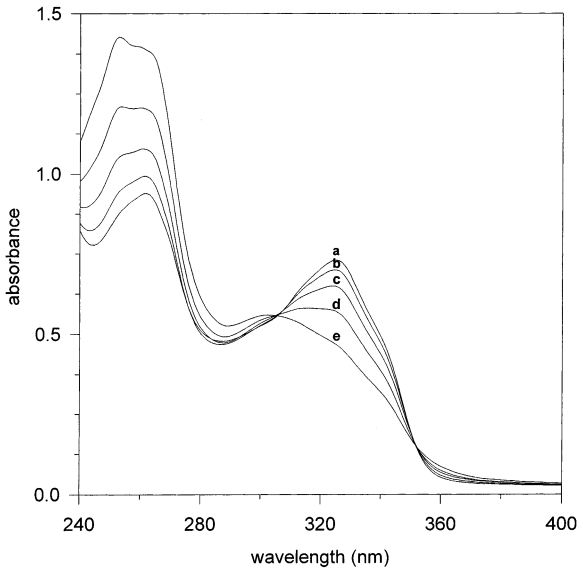


Figure 2. UV absorption spectra of an ethanol solution of furan cycloadduct obtained from salmon testes DNA and **6** before (line a) and after irradiation at 254 nm (10, 30, 60, 120 min, lines b−e, respectively).

in the hydrogenated fourth ring does not counteract the capacity of the involved double bond to photoreact.

To evaluate the covalent photobinding process of **6** and **9**, it appeared interesting to analyze the 4′,5′-photoadduct formation.

The irradiation (365 nm) of an aqueous solution of DNA in the presence of the two considered compounds, followed by precipitation and acid hydrolysis, as reported in the Experimental Section, allows us to isolate a product characterized by a strong violet fluorescence. This property is usually consistent with the molecular structure of the furan side monoadduct.[19] Figure 2 shows the UV spectrum of an ethanol solution of the fluorescent compound obtained by the photoreaction between **6** and DNA (line a). It can be observed that its trend is similar to that already observed for other C_4-cycloadducts between the furan double bond of the furocoumarins and the 5,6-double bond of pyrimidine:[20] an evident absorption at 330 nm appears, while the peculiar furocoumarin band around 300 nm disappears. To provide additional evidence, photoreversion experiments were performed. Indeed, it is known that, when irradiated at 254 nm, the C_4-cycloadducts undergo breakage, yielding the parent compounds, i.e., the furocoumarin, and the DNA base. Figure 2 also reports the UV absorption spectra of the photoproduct after increasing periods of irradiation at 254 nm (lines b−e). After 2 h, the recorded spectrum shows the disappearance of the peak at 330 nm and the occurrence of the absorption band at 301 nm, typical of **6** (line e). Similar results have been obtained also for **9** (data not shown).

Nevertheless, confirmation of the above assumption was obtained after the characterization of the isolated photoproducts.

As regards the fluorescent compound isolated from the photoreaction between **6** and DNA, Figure 3 reports the mass spectrum where the peak at *m*/*z* 482 is consistent with a thymine−**6** photoadduct. To characterize the structure of the photoproduct, ^{1}H and ^{13}C NMR spectra were performed. With regard to the ^{1}H spectrum (Figure 4), a doublet attributed to HC-5′ at δ 5.13 with a ^3J = 5.25 Hz due to its coupling with the thymine HC-6 appears, which is evidence for the photoadduct formation between tetrahydrobenzopsoralen derivative **6** and the pyrimidine. The HC-6 signal occurs at δ 3.88. Furthermore, the shift of a furan methyl singlet from δ 2.23 in the spectrum of **6** to δ 1.42 and that of a thymine methyl singlet from δ 1.63 in the spectrum of thymine to δ 1.46, that is, toward the aliphatic region, represents a further confirmation of the occurrence of photoaddition, since it shows that the furan ring added thymine by its 4′,5′-double bond, yielding a no more aromatic system. Moreover, to assign the ^{13}C spectrum, obtain bond connectivities, and establish the multiple bond proton−carbon connectivities, ^{1}H−^{13}C HMQC and HMBC spectra were obtained. In particular, the presence of four saturated carbon signals (δ 44.9, 53.3, 54.3, and 85.1) that are assignable to the carbons 5 and 6 of the thymine and 4′ and 5′ of the furan ring is consistent with the occurrence of a C_4-cycloadduct between the 4′,5′-double bond of **6** and the 5,6-double bond of thymine. Finally, the confirmation of the photoadduct structure derives from HMBC analysis (see the inset in Figure 4), which also allows us to assign to the isolated thymine−**6** photoadduct, in agreement with previous isolated furan side adducts, a cis configuration.[20,21] This means that, upon intercalation, **6** photoadds to thymine in such a way that the psoralen moiety and the DNA base lie on the same side of the cyclobutane plane.

The fluorescent photoproduct isolated from the photoreaction between DNA and **9** was analyzed by mass spectrometry, and the resulted spectrum is reported in Figure 5, where the major peak, which appears at *m*/*z* 478, corresponds to the thymine−**9** C_4-cycloadduct.

Cross-Linking. A peculiar property of the furan side monoadduct is the possibility to absorb a second photon (365 nm) to photoreact also at the 3,4-double bond, thus giving rise to an interstrand cross-link with a pyrimidine belonging to the opposite DNA strand. As already stated, the condensation of a cyclohexenyl ring to the 4′,5′-photoreactive furan side allowed the photoaddition to the same double bond. Consequently, they have been demonstrated to behave as bifunctional compounds. On the contrary, for the analogous tetracyclic benzoderivatives the aromatic fourth ring at the level of the furan double bond prevents the photoaddition, rendering these compounds monofunctional molecules.[12,14]

Also for the new tetracyclic benzopsoralens **7**−**9**, the inability to give rise to diadducts with the macromolecule has been confirmed (data not shown). In this case the fourth ring at the pyrone side did not allow the furan monoadduct to photoreact further. Regarding the tetrahydrobenzo congeners **4**−**6**, Figure 6 shows the results obtained by denaturation−renaturation experiments, in comparison with 8-MOP. For all the new tetrahydrobenzopsoralens, a certain ability to form cross-links has been detected that decreases in the following order: **6** > **4** > **5**. Nevertheless, this ability appears to be lower with respect to that obtained for the reference drug.

Considering **6** and **9**, the comparison between the above data and the photoantiproliferative activity reported in Table 1, suggests that for these moieties the

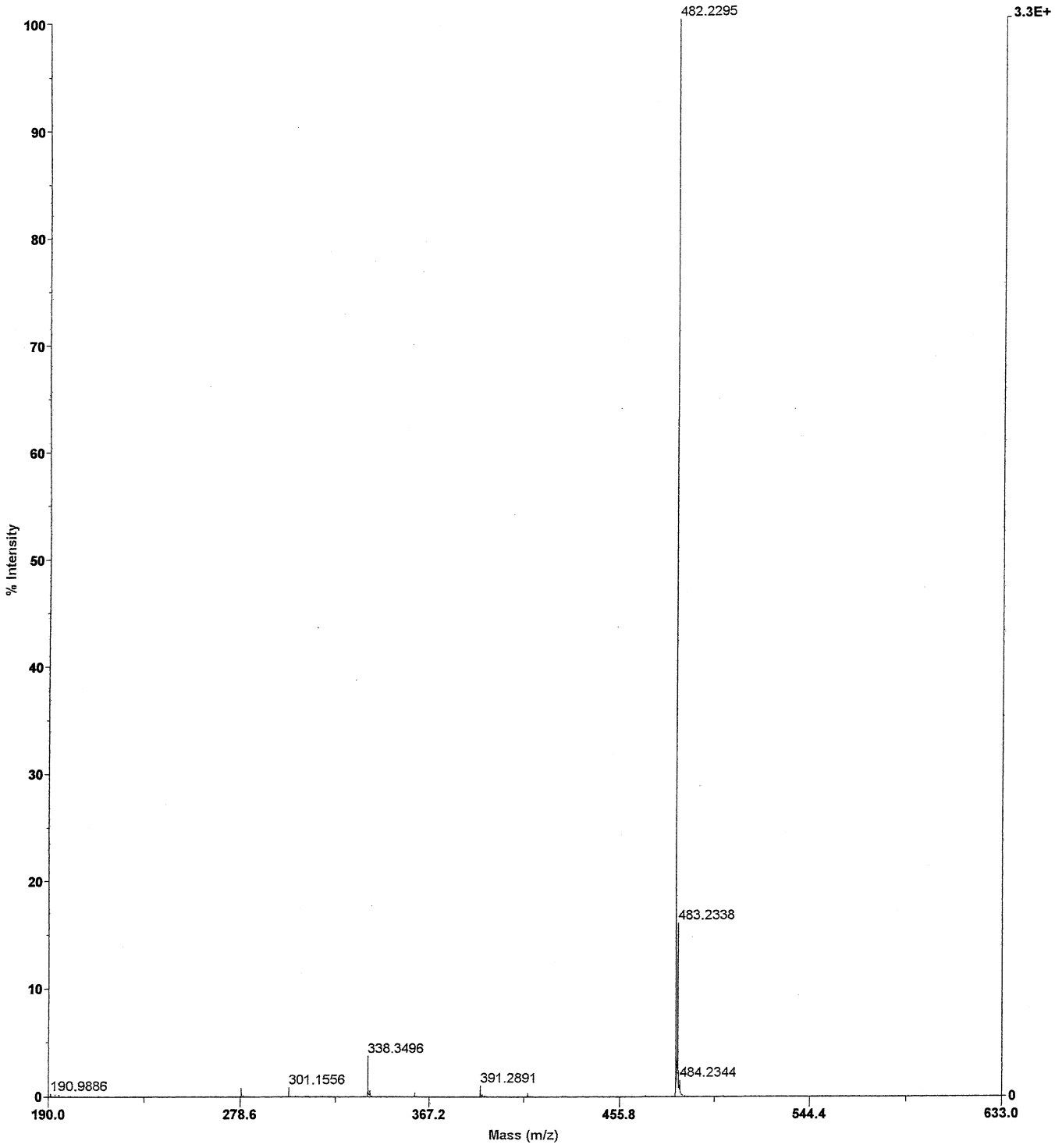


Figure 3. Mass spectrum of the **6**−thymine photoadduct.

ability to induce bifunctional damage to the DNA does not seem to be determinant for the cytotoxic effect, probably also in connection with the very moderate amount of cross-links induced by **6**.

In a previous study, for a tetrahydrobenzopsoralen analogue to **6**, which differs from the latter by the condensation of the fourth ring at the 4′,5′-double bond rather than at the 3,4-double bond, we demonstrated a higher capacity to form cross-links versus 8-MOP.[14]

On the basis of the overall obtained results, it is possible to affirm that the condensation of the cyclohexenyl ring at the 3,4-pyrone side seems to inhibit the ability of the tetracyclic moiety to form cross-links with respect to the condensation at the 4′,5′-furan side.

Theoretical Calculations. To investigate more in depth the different photo-cross-linking ability of **6** with respect to its congener characterized by having the fourth cyclohexenyl ring condensed at the 4′,5′-photoreactive double bond, molecular modeling studies were carried out. For the sake of completeness, in these studies **9** and its congener with the benzene ring condensed at the 4′,5′-double bond[14] were also included.

As a first step, a molecular geometry optimization at the AM1 level on all compounds was performed (Figure

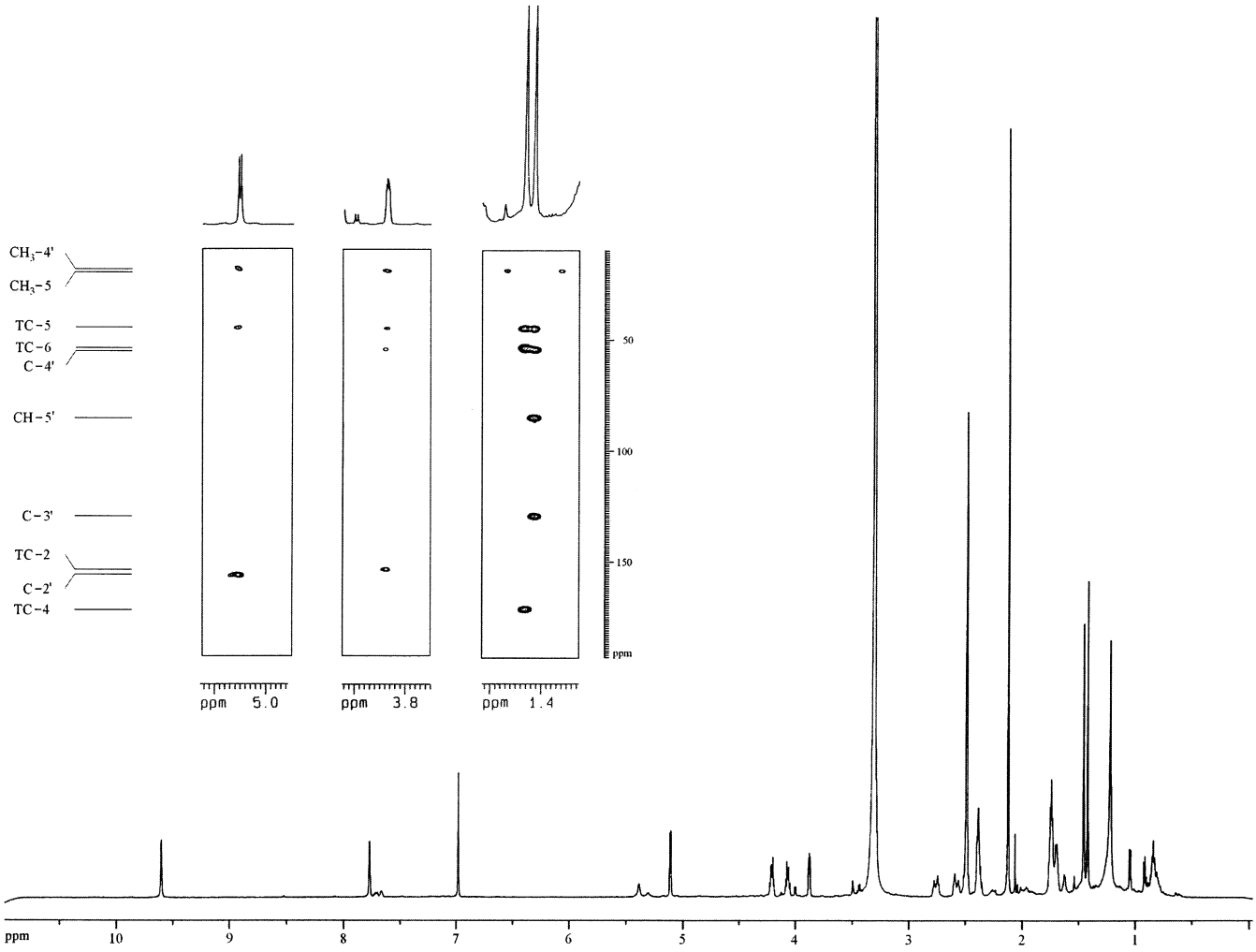


Figure 4. ^{1}H NMR spectrum of the **6**−thymine furan photoadduct. In the inset relevant HMBC spectrum regions are reported.

7). All molecules show the tetracyclic skeleton to be flat except for the two cyclohexene carbons farthest from the rest of the system, which can lie alternatively above and below the chromophore mean plane, thus originating two possible conformations. For **6**, such conformations (**I**, **II**) have practically equal energies of about −93 kcal/mol. The two corresponding conformations of its congener (**III**, **IV**) behave similarly and have also equal energies of about −91 kcal/mol (that is, 2 kcal/mol higher in energy). The displacement of the cyclohexene carbons in the two molecules from the plane of the tetracycle was within 0.42 Å in **I** and **II** and within 0.37 Å in **III** and **IV**. With respect to the benzo derivatives, the optimized geometry of **9** (**V**) has an energy of −59.4 kcal/mol, while its congener (**VI**) has an energy of −56.2 kcal/mol.

It is known that the occurrence of a covalent photoaddition to the double helix requires, in the first place, an effective intercalation complex between two base pairs. To compare the ability of the molecules taken into account to fit into a model of their binding site, a very simple representation of the DNA intercalation site was prepared by modeling in vacuo a tetranucleotide, $d(TA)_2$, where the two base pairs were positioned as described in previous studies.[22] Also the position of the compounds (**I**−**VI**) was chosen in such a way as to reproduce the same arrangement of the intercalating compound described in such a study.[22] Accordingly, the dimethylaminopropyl tail of all the molecules ends up in the major groove. The intercalation site was also geometrically optimized at the AM1 level. Figure 8 shows a representation of the interaction of **6** with the $d(TA)_2$ tetranucleotide.

Finally, the energies of the interaction of **I**−**VI** with the $d(TA)_2$ tetranucleotide were investigated. The complexes showed relative energies of 2.98 (**I**), 6.51 (**II**), 0.00 (**III**), 3.51 (**IV**), 30.7 (**V**), and 30.7 (**VI**) kcal/mol, respectively. The values obtained for the tetrahydrobenzo derivatives (**I**−**IV**) suggest that the condensation of the fourth ring at the 4′,5′-double bond renders the psoralen moiety better suited to fit into the intercalation pocket with respect to the condensation at the 3,4-site. Thus, the more unfavorable interaction energy of **6** could account for the resulting decrease in its ability to form cross-links when compared with the congener.

With respect to **V** and **VI**, the energy of the complexes are the same, suggesting that no significant difference exists between the two benzo derivatives. The energies of the interaction of **V** and **VI** with the tetranucleotide are much higher than those shown by **I**−**IV**. Nevertheless, it has to be underlined that they appear to be mainly determined by the energies of the two benzo derivative moieties alone.

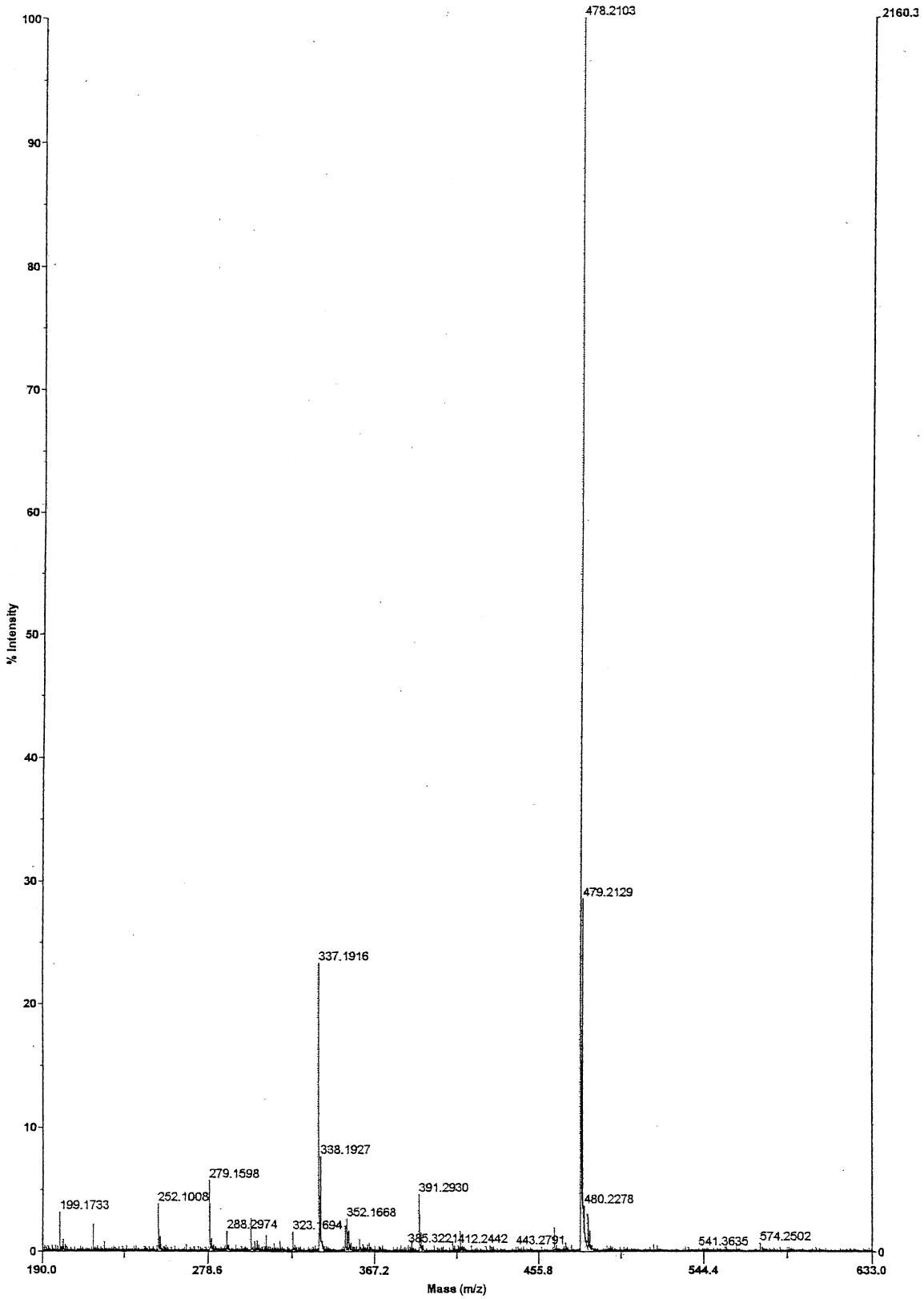


Figure 5. Mass spectrum of the **9**−thymine photoadduct.

Conclusions

New tetracyclic psoralen derivatives were synthesized and their photobiological behavior studied. These compounds are characterized by the condensation of a benzenic or a cyclohexenyl ring at the photoreactive 3,4-double bond of the psoralen moiety. Furthermore, in both series of tetracyclic derivatives, a methoxy, a hydroxy, or a dimethylaminopropoxy side chain was inserted at position 11 of the chromophore.

The evaluation of the antiproliferative activity on human tumor cell lines after UVA irradiation evidenced a remarkable effect for the new compounds bearing the

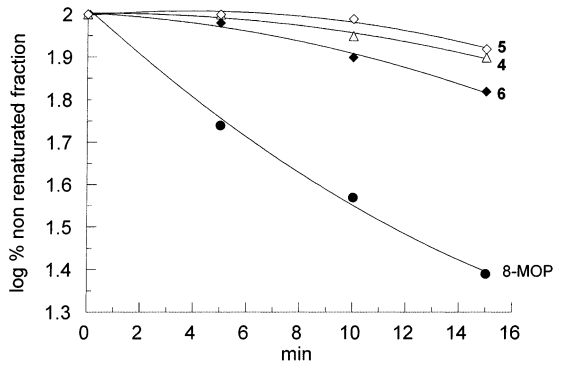


Figure 6. Cross-linking of compounds **4**−**6** and 8-MOP to double-stranded DNA from salmon testes (nucleotide/drug ratio = 75) as a function of irradiation time.

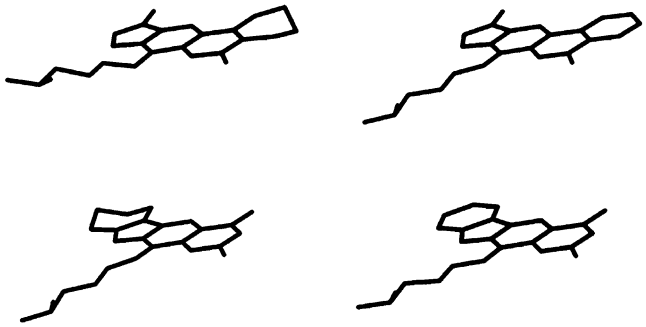


Figure 7. A representation of **6** (top left), **9** (top, right), and their furan side congeners.

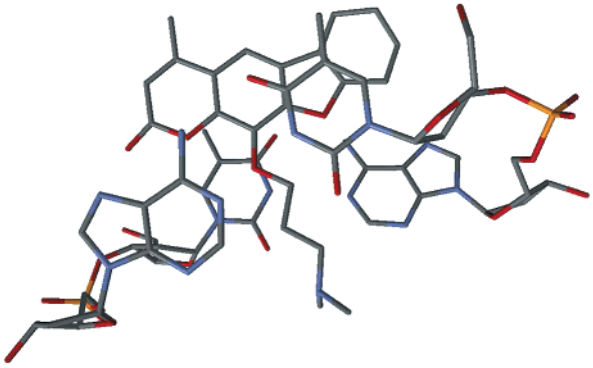


Figure 8. A representation of the interaction of **6** with the $d(TA)_2$ tetranucleotide.

dimethylaminopropoxy side chain, both tetrahydrobenzo (compound **6**) and benzo (compound **9**). Indeed, they are able to exert a cytotoxic ability from 5 to 15 times higher with respect to that obtained for the reference drug 8-MOP. Otherwise, the activity scored for the synthesized compounds carrying the hydroxy or the methoxy group appeared to be lower than that of 8-MOP or, in some cases, even absent at the tested concentrations. The evaluation of the appearance of erythema, a marker of cutaneous photosensitization, increased the interest in derivatives **6** and **9**, due to the fact that both appear to be clearly nonphototoxic. The insertion of a fourth ring can be considered responsible for having conferred this helpful feature. In this regard, taking into consideration a previous study[14] in which analogues to **6** and **9** characterized by the fourth ring condensed at the 4′,5′-double bond were extensively studied, some considerations can be made. It can be noted that, while for both benzopsoralen derivatives a lack of skin photosensitizing ability has been found, a different pattern appears in the case of the tetrahydrobenzopsoralen congeners. In more detail, the skin phototoxicity was decreased with respect to the reference drug by the condensation of the fourth cyclohexenyl ring at the 4′,5′-double bond of psoralen moiety, but it is completely abolished if the condensation takes place at the pyrone side. Furthermore, it is worth underlining the fact that for both tetrahydrobenzopsoralen congeners, the antiproliferative activity is comparable and significantly higher with respect to 8-MOP.

The study of the interaction of both **6** and **9** with DNA in the ground state and after UVA irradiation indicated that the behavior already demonstrated for the psoralens occurred. In particular, they form a complex with DNA via an intercalative mode of binding and, after irradiation, are able to photoadd to the macromolecule. Specifically, the C_4-cycloadducts involving the 4′,5′-double bond of the psoralen chromophore and the 5,6-double bond of thymine were isolated and characterized by spectroscopic techniques.

For tetrahydrobenzopsoralen derivatives **4**−**6**, the ability to form interstrand cross-links has been demonstrated, even though it is lower with respect to that found for 8-MOP. The comparison with the analogue of **6** endowed with the fourth cyclohexenyl ring at the 4′,5′-double bond highlighted the differing capacities of the two congeners to induce cross-links in the macromolecule. In detail, the amount induced by **6** appears to be significantly lower with respect to that obtained for its analogue. The energy profile of the two compounds, derived from theoretical calculations, accounted for the observed behaviors. In fact a more unfavorable interaction energy was detected for **6**, and this seems to account for the more difficult subsequent cycloaddition reaction.

In conclusion, the photobiological properties of compounds **6** and **9** allow us to consider the new benzo- and tetrahydrobenzotetracyclic moieties as an interesting model in the development of new photochemotherapeutic drugs. Moreover, a further reason for the interest in **6** lies in the possibility that it allows correlation between chemical structures with the aim of better delineating the structure−activity relationships that regulate the photobiological effects of PUVA therapy.

Experimental Section

Melting points are uncorrected and were determined with a Reichert Kofler thermopan or in capillary tubes in a Büchi 510 apparatus. IR spectra were recorded with a Perkin-Elmer 1640FT spectrometer (KBr disks, υ in cm^{-1}). ^1H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra were recorded with a Bruker AMX spectrometer, using TMS as internal standard (chemical shifts in δ values, J in hertz). Mass spectrometry was carried out with a Kratos MS-50 or a Varian MAT-711 spectrometer. Elemental analyses were performed by a Perkin-Elmer 240B microanalyzer and were within ±0.4% of calculated values in all cases. Flash chromatography (FC) was performed on silica gel (Merck 60, 230−400 mesh); analytical TLC was performed on precoated silica gel plates (Merck 60 F_{254}, 0.25 mm).

3,4-Cyclohexene-7-hydroxy-8-methoxycoumarin (2). Ethyl 2-oxocyclohexanecarboxylate (16.39 mL, 17.49 g, 102.76 mmol) was added to 2-methoxyresorcinol (**1**, 12.0 g, 85.63 mmol) in concentrated H_2SO_4 (120 mL) at 0 °C. The mixture was stirred for 10 h at room temperature, poured into ice−water (500 mL), and left overnight. A yellow precipitate was

recovered by filtration, washed with water, and purified by FC with 1:1 hexane−ethyl acetate as eluent, giving pure **2** (19.55 g, 93%). Mp: 182−183 °C. IR: 3363, 2941, 1689, 1577, 1315, 1092, 798. ^{1}H NMR (DMSO-d_6): 1.72 (m, 4H, $CH_2(CH_2)_2CH_2$), 2.38 (m, 2H, CH_2 in 4), 2.70 (m, 2H, CH_2 in 3), 3.81 (s, 3H, CH_3O), 6.85 (d, J = 8.80, 1H, H-6), 7.26 (d, J = 8.80, 1H, H-5), 10.16 (bs, 1H, HO). ^{13}C NMR (DMSO-d_6): 20.77 (CH_2), 21.11 (CH_2), 23.39 (CH_2), 24.61 (CH_2), 60.46 (CH_3), 112.76 (CH), 118.25 (C), 118.43 (C), 118.68(CH), 133.89 (C), 145.88 (C), 147.83 (C), 152.23 (C), 160.51(C). MS *m*/*z* (%): 247 ([M + 1]$^+$, 67), 246 (M$^+$, 100), 230 (44), 218 (42), 190 (68), 157 (14). Anal. $C_{14}H_{14}O_4$: C, H.

7-Acetonyloxy-3,4-cyclohexene-8-methoxycoumarin (3). To a solution of **2** (19.27 g, 78.25 mmol) in dry acetone (370 mL) were added chloroacetone (14.48 g, 156.50 mmol) and K_2CO_3 (21.63 g, 156.50 mmol), and the reaction mixture was refluxed for 24 h. The precipitate was filtered out and the filtrate was concentrated under vacuum to a solid residue that was purified by FC with 6:4 hexane−ethyl acetate as eluent, giving pure **3** (16.85 g, 71%). Mp: 141−143 °C. IR: 2941, 1734, 1697, 1609, 1302, 1115, 795. ^{1}H NMR ($CDCl_3$): 1.82 (m, 4H, $CH_2(CH_2)_2CH_2$), 2.31 (s, 3H, CH_3CO), 2.55 (m, 2H, CH_2 in 4), 2.72 (m, 2H, CH_2 in 3), 4.01 (s, 3H, CH_3O), 4.70 (s, 2H, CH_2O), 6.73 (d, J = 8.90, 1H, H-6), 7.22 (d, J = 8.90, 1H, H-5). ^{13}C NMR ($CDCl_3$): 21.90 (CH_2), 22.18 (CH_2), 24.53 (CH_2), 25.86 (CH_2), 27.12 (CH_3), 62.23 (CH_3), 74.67 (CH_2), 110.50 (CH), 116.66 (C), 118.65 (CH), 122.22 (C), 137.19 (C), 147.03 (C), 147.70 (C), 152.83 (C), 161.85 (C), 205.22 (C). MS *m*/*z* (%): 302 (M$^+$, 100), 259 (26), 245 (55), 214 (17), 128 (14), 115 (17). Anal. $C_{17}H_{18}O_5$: C, H.

3,4-Cyclohexene-4′-methyl-8-methoxyfuro[3,2-*g*]coumarin (4). A mixture of **3** (16.16 g, 53.45 mmol) and 1 M NaOH (280 mL) was refluxed for 24 h, cooled, and acidified with 3 M HCl. The precipitate was recovered by filtration, washed with water, and purified by FC with 9:1 hexane−ethyl acetate as eluent, yielding pure **4** (12.45 g, 82%). Mp: 204−205 °C. IR: 3112, 2945, 1711, 1623, 1590, 1343, 1158, 827. ^{1}H NMR ($CDCl_3$): 1.84 (m, 4H, $CH_2(CH_2)_2CH_2$), 2.25 (d, J = 1.25, 3H, CH_3), 2.57 (m, 2H, CH_2 in 4), 2.83 (m, 2H, CH_2 in 3), 4.25 (s, 3H, CH_3O), 7.25 (s, 1H, H-5), 7.42 (d, J = 1.25, 1H, H-5′). ^{13}C NMR ($CDCl_3$): 7.74 (CH_3), 21.38 (CH_2), 21.53 (CH_2), 23.96 (CH_2), 25.67 (CH_2), 61.14 (CH_3), 106.08 (CH), 115.84 (C), 117.33 (C), 121.60 (C), 127.10 (C), 132.16 (C), 141.28 (C), 142.43 (CH), 146.53 (C), 147.45 (C), 161.38 (C). MS *m*/*z* (%): 285 ([M + 1]$^+$, 18), 284 (M$^+$, 100), 256 (19), 228 (52), 191 (5), 128 (8). Anal. $C_{17}H_{16}O_4$: C, H.

3,4-Cyclohexene-8-hydroxy-4′-methylfuro[3,2-*g*]coumarin (5). A mixture of $AlCl_3$ (844 mg, 6.331 mmol) and dry CH_2Cl_2 (25 mL) was stirred for 2 h at room temperature. A solution of compound **4** (600 mg, 2.11 mmol) in dry CH_2Cl_2 (15 mL) was added, and stirring was continued for another 3 h. The reaction mixture was then acidified with HCl and extracted with CH_2Cl_2 (4 × 100 mL), the extract was dried (Na_2SO_4), and the solvent was evaporated under reduced pressure, leaving a residue that upon purification by FC with 98:2 CH_2Cl_2/MeOH as eluent yielded pure **5** (497 mg, 87%). Mp: 312−314 °C. IR: 3306, 2956, 1696, 1592, 1290, 1112, 824. ^{1}H NMR (DMSO-d_6): 1.77 (m, 4H, $CH_2(CH_2)_2CH_2$), 2.22 (d, J = 1.30, 3H, CH_3), 2.43 (m, 2H, CH_2 in 4), 2.86 (m, 2H, CH_2 in 3), 7.33 (s, 1H, H-5), 7.80 (d, J = 1.30, 1H, H-5′), 10.41 (bs, 1H, HO). ^{13}C NMR (DMSO-d_6): 7.45 (CH_3), 20.84 (CH_2), 21.08 (CH_2), 23.60 (CH_2), 25.09 (CH_2), 103.50 (CH), 115.63 (C), 116.64 (C), 120.19 (C), 126.13 (C), 129.48 (C), 138.13 (C), 143.04 (CH), 144.30 (C), 148.05 (C), 160.53 (C). MS *m*/*z* (%): 271 ([M + 1]$^+$, 18), 270 (M$^+$, 100), 242 (53), 227 (28), 214 (74), 201 (20), 177 (19), 115 (17). Anal. $C_{16}H_{14}O_4$: C, H.

3,4-Cyclohexene-8-(3-dimethylaminopropoxy)-4′-methylfuro[3,2-*g*]coumarin (6). A mixture of hydroxytetrahydrobenzofurocoumarin **5** (229.0 mg, 0.847 mmol), 3-chloro-*N*,*N*-dimethylpropylamine hydrochloride (160 mg, 1.016 mmol), 60% NaH (61 mg, 2.541 mmol), NaI (152 mg, 1.016 mmol), and dimethylformamide (25 mL) was heated for 4 h at 100 °C and then concentrated under reduced pressure. The residue was purified by FC with 98:2 CH_2Cl_2–methanol as eluent and then treated with ion-exchange resin IRA-4000, giving pure **6** (185 mg, 62%). Mp: 112−113 °C. IR: 3418, 2938, 1705, 1591, 1400, 1115, 753. ^{1}H NMR (DMSO-d_6): 1.78 (m, 4H, $CH_2(CH_2)_2CH_2$), 1.85 (m, 2H, $CH_2CH_2CH_2$), 2.12 (s, 6H, $(CH_3)_2N$), 2.23 (d, J = 1.30, 3H, CH_3 in 4′), 2.43 (t, J = 7.00, 2H, CH_2N), 2.43 (m, 2H, CH_2 in 4), 2.88 (m, 2H, CH_2 in 3), 4.40 (t, J = 6.40, 2H, CH_2O), 7.56 (s, 1H, H-5), 7.85 (d, J = 1.30, 1H, H-5′). ^{13}C NMR (DMSO-d_6): 7.29 (CH_3), 20.76 (CH_2), 20.98 (CH_2), 23.58 (CH_2), 25.02 (CH_2), 27.65 (CH_2), 45.10 (2 CH_3), 55.35 (CH_2), 71.50 (CH_2), 107.08 (CH), 115.61 (C), 116.69 (C), 120.43 (C), 126.56 (C), 130.37 (C), 140.99 (C), 143.27 (CH), 146.20 (C), 147.60 (C), 160.21 (C). MS *m*/*z* (%): 356 ([M + 1]$^+$, 10), 355 (M$^+$, 44), 310 (4), 270 (16), 214 (12), 58 (100). Anal. $C_{21}H_{25}NO_4$: C, H, N.

3,4-Benzo-4′-methyl-8-methoxyfuro[3,2-*g*]coumarin (7). A solution of tetrahydrobenzopsoralen **4** (1.0 g, 3.517 mmol) and DDQ (0.798 g, 3.517 mmol) in toluene (80 mL) was refluxed for 6 h. The mixture was cooled, the precipitate collected, the solvent evaporated under reduced pressure, and the resulting residue purified by FC with CH_2Cl_2 as eluent, giving pure **7** (591 mg, 60%). Mp: 193−194 °C. IR: 2946, 1713, 1600, 1386, 1245, 1115, 840. ^{1}H NMR ($CDCl_3$): 2.27 (d, J = 1.30, 3H, CH_3), 4.27 (s, 3H, CH_3O), 7.40 (bs, 1H, H-5′), 7.49 (m, 1H, C*H*−CH−C3), 7.68 (s, 1H, H-5), 7.77 (m, 1H, C*H*−CH−C4), 8.09 (m, 1H, CH−C4), 8.31 (m, 1H, CH−C3). ^{13}C NMR ($CDCl_3$): 7.85 (CH_3), 61.22 (CH_3), 105.57 (CH), 115.03 (CH), 115.83 (C), 120.19 (C), 121.87 (CH), 127.70 (C), 128.25 (CH), 130.60 (CH), 133.16 (C), 134.74 (CH), 134.87 (CH), 135.61 (C), 142.72 (CH), 146.62 (C), 160.86 (C). MS *m*/*z* (%): 281 ([M + 1]$^+$, 18), 280 (M$^+$, 100), 237 (70), 209 (7), 181 (10), 152 (32). Anal. $C_{17}H_{12}O_4$: C, H.

3,4-Benzo-8-hydroxy-4′-methylfuro[3,2-*g*]coumarin (8). This compound was prepared from **7** (712 mg) in the same way as **5** from **4**. The crude product was purified by FC with 99:1 methylene chloride−methanol as eluent, yielding pure **8** (525 mg, 78%). Mp: 288 °C (dec). IR: 3385, 2923, 1713, 1605, 1302, 1121, 766. ^{1}H NMR (DMSO-d_6): 2.28 (d, J = 1.10, 3H, CH_3), 7.63 (m, 1H, C*H*−CH−C3), 7.83 (d, J = 1.10, 1H, H-5′), 7.93 (m, 1H, C*H*−CH−C4), 8.02 (s, 1H, H-5), 8.24 (dd, J = 0.80 and 7.84, 1H, CH−C4), 8.50 (m, 1H, CH−C3), 10.51 (bs, 1H, HO). ^{13}C NMR (DMSO-d_6): 7.52 (CH_3), 103.34 (CH), 114.43 (C), 115.69 (C), 119.31 (C), 122.80 (CH), 126.64 (C), 128.32 (CH), 129.56 (CH), 130.32 (C), 135.06 (CH), 135.52 (C), 137.54 (C), 143.15 (CH), 144.36 (C), 160.17 (C). MS *m*/*z* (%): 267 ([M + 1]$^+$, 17), 266 (M$^+$, 100), 237 (8), 210 (7), 181 (16), 152 (12). Anal. $C_{16}H_{10}O_4$: C, H.

3,4-Benzo-8-(3-dimethylaminopropoxy)-4′-methylfuro-[3,2-*g*]coumarin (9). This compound was prepared from **8** (150.0 mg) in the same way as **6** from **5**. The crude product was purified by FC with 98:2 methylene chloride−methanol as eluent, giving pure **9**·HCl (146 mg, 67%). Mp: 237 °C. IR: 2923, 2700, 1720, 1475, 1298, 1115, 769. ^{1}H NMR (DMSO-d_6): 2.14 (m, 2H, $CH_2CH_2CH_2$), 2.30 (d, J = 1.15, 3H, CH_3 in 4′), 2.87 (s, 6H, $(CH_3)_2N$), 3.41 (m, 2H, CH_2N), 4.48 (t, J = 5.75, 2H, CH_2O), 7.70 (m, 1H, C*H*−CH−C3), 7.91 (d, 1H, J = 1.15, H-5′), 7.97 (dt, 1H, J = 7.80 and 1.20, C*H*−CH−C4), 8.25 (dd, 1H, J = 7.80 and 1.10, CH−C4), 8.33 (s, 1H, H-5), 8.57 (m, 1H, CH−C3). ^{13}C NMR (DMSO-d_6): 7.47 (CH_3), 24.73 (CH_2), 42.52 (2 CH_3), 54.37 (CH_2), 70.49 (CH_2), 107.98 (CH), 114.76 (C), 115.85 (C), 119.37 (C), 122.93 (CH), 127.22 (C), 128.69 (CH), 129.55 (CH), 130.54 (C), 135.09 (C), 135.23 (CH), 140.75 (C), 143.60 (CH), 146.30 (C), 160.01 (C). MS *m*/*z* (%): 352 ([M + 1]$^+$, 9), 351 (M$^+$, 40), 278 (5), 266 (7), 250 (7), 237 (21), 181 (14), 152 (45), 58 (100). Anal. $C_{21}H_{22}ClNO_4$: C, H, Cl, N.

Photobiological Methods. Cell Cultures. HL-60 and HeLa cells were grown in RPMI 1640 (Sigma Chemical Co.) supplemented with 15% heat-inactivated fetal calf serum (Seromed) and Nutrient Mixture F-12 [HAM] (Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal calf serum (Seromed), respectively. Penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) (Sigma Chemical Co.) were added to both media. The cells were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air.

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

Inhibition Growth Assays. HeLa cells (10^5) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, the medium was replaced with an equal volume of Dulbecco's modified Eagle medium (DMEM, Sigma Chemical Co.) without phenol red, and various concentrations of the test agent were added. One hour later the cells were irradiated with a UVA dose of 0.793 J cm^{-2}. After irradiation, the medium containing the compounds was removed, and the cells were incubated in complete F-12 medium for 24 h. In the case of the experiments carried out in the dark, the replacement of the incubation medium was omitted and the cells were incubated in complete F-12 medium in the presence of the test compound for 24 h.

HL-60 cells (10^5) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, various concentrations of the test agents were added in complete medium. The cells were kept in the dark for 1 h, irradiated with a UVA dose of 0.793 J cm^{-2}, and then incubated for a further 24 h.

A trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as IC_{50} values, i.e., the concentrations of the test agent inducing 50% reduction in cell numbers compared with control cultures.

Skin Phototoxicity. Skin phototoxicity was tested on depilated albino guinea pigs (outbred Dunkin−Hartley strain), as previously reported.[20] An ethanol solution of each new compound was applied topically to the skin up to 50 $\mu g/cm^2$. For 8-MOP, the concentration used was 10 $\mu g/cm^2$. The animals were then kept in the dark for 45 min and the treated skin was irradiated with 20 kJ m^{-2} of UVA; erythema was scored after 48 h.

Nucleic Acid. Salmon testes DNA was purchased from Sigma Chemical Co. (Cat. D-1626). Its hypochromicity, determined according to the method of Marmur and Doty,[23] was over 35%. The DNA concentration was determined using extinction coefficient 6600 $M^{-1}cm^{-1}$ at 260 nm.

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

Linear dichroism is defined as

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

where $A_{\parallel}$ and $A_{\perp}$ correspond to the absorbances of the sample when polarized light is oriented parallel or perpendicular to the flow direction, respectively. The orientation is produced by a device designed by Wada and Kozawa[24] at a shear gradient of 500−700 rpm and each spectrum was accumulated four times.

A solution of salmon testes DNA (1.5×10^{-3}M) in ETN buffer (containing 10 mM TRIS, 10 mM NaCl, and 1 mM EDTA, pH = 7) was used. Spectra were recorded at 25 °C at different [DNA]/[drug] ratios.

Preparation of Adducts. Volumes of concentrated solutions of the examined compound were added to salmon testes DNA in ETN solution (1.5×10^{-3}M) to achieve a DNA/compound ratio of about 80. The mixture was irradiated in a glass dish with four Philips HPW 125 lamps, arranged two above and two below the dish, at a distance of 7 cm, for 120 min at room temperature. After irradiation the DNA was precipitated with NaCl (up to 1 M concentration) and cool ethanol (2 volumes), and the precipitated DNA was collected, washed with 80% ethanol, dried, and then dissolved in a measured volume of buffer. The final solution was made 0.5 N with HCl, heated at 100 °C for 2 h, neutralized, and extracted exhaustively with $CHCl_3$. After this procedure the organic layers were collected, dried under high vacuum, and dissolved in ethanol, and the adduct was separated on TLC plates and eluted with $CHCl_3$/ethanol/ammonium hydroxide 9:0.8:0.2. UV spectra were recorded on a Perkin-Elmer model Lambda 5 spectrophotometer. The ^1H and ^{13}C assignment was obtained by utilizing HMQC and HMBC spectra on a Bruker Avance DMX600 instrument. Mass spectrometry measurements were performed on a ElectroSpray Ionization (ESI) time-of-flight (ToF) instrument (model Mariner, Perseptive-Biosystem) by dissolving the samples in water/acetonitrile/formic acid (50:49:1) solution.

Photoreversal of Adducts. An ethanol solution of the adduct (ca. 20 μg/mL) was irradiated in quartz cuvettes with a mineral lamp (254 nm). The photosplitting reaction was followed spectrophotometrically.

Evaluation of Interstrand Cross-Links in Vitro. Evaluation of cross-links was carried out by measuring the renaturation capacity of cross-linked DNA after thermal denaturation. Aliquots of a solution of DNA and examined compound [DNA]/[drug] = 75 were introduced into calibrated glass tubes, immersed in a thermostatically controlled bath, and then irradiated for various periods of time. After irradiation the samples were thermally denatured (95 °C for 15 min) and quickly cooled in ice. The renaturation capacity of DNA, due to cross-link formation, was investigated by recording absorbance at 260 nm. Data were expressed in terms of log % of nonrenaturated fraction of irradiated compound−DNA complex relative to irradiated DNA, as suggested by Blais et al.[25]

Theoretical Calculations. Theoretical optimization of the molecular geometries, followed by manual docking into a DNA binding site model was calculated at the AM1 semiempirical quantum mechanical level,[26] using the AM1 module of the PC Spartan Pro program[27] run on an SGI 320 workstation.

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