Furocoumarin−Oligonucleotide Interaction: Kinetics, Selectivity, and Mechanism of the Furocoumarin Photoaddition Reaction to Oligonucleotide Intercalation Sites

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The interaction and subsequent photoaddition reaction on UVA irradiation of 4,4′-dimethyl-5-methoxyangelicin and 4,4′-dimethyl-5-methoxypsoralen to twelve synthetic oligonucleotides was studied. A new approach was developed which allows: (a) accurate determination of the kinetics constant of the overall photoaddition process to each oligonucleotide; (b) identification of the different types of intercalation sites, individualized by a combination of two consecutive base pairs and the frequency of their appearance along the oligomer sequences; and (c) the value of the kinetics constant relative to each type of site to be determined. This approach also makes it possible to highlight the influence of sequences flanking each site that participate in the photoaddition process. Thus, the possibility of discriminating which sites are active in the photoaddition reaction and their contribution to the overall process appears to be a useful tool for studying the correlation between furocoumarin photocombination with DNA and the consequent biological effects.

Introduction

Furocoumarins are an important class of both linear (psoralens) and angular (such as angelicins) compounds that exert photobiological effects after exposure with UVA light (320−400 nm).[1,2] Among them, 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and 4,5′,8-trimethylpsoralen are widely used in PUVA (psoralen plus ultraviolet A radiation) therapy for the treatment of some skin diseases, such as psoriasis, that is, a hyperproliferative disease, and vitiligo, that is, an acquired pigmentary alteration.[3,4]

A development of PUVA leads to photopheresis, an extracorporeal form of photochemotherapy. By means of photopheresis, 8-MOP is used for the treatment of cutaneous T-cell lymphoma.[5] Furthermore, encouraging results have been obtained by using photopheresis to prevent rejection of heart transplants, in the treatment of AIDS-related complexes and in several autoimmune diseases such as phemphigus vulgaris, systemic sclerosis, rheumatoid arthritis, and systemic lupus erythematosus.[6]

DNA damage is considered to play an important role in the mechanism of furocoumarins action.[7] The photoreaction of furocoumarins with DNA is the final result of a multistage process. The initial step is formation of an intercalative complex between the furocoumarin and the nucleic acid helix in a dark reaction. On exposure to UVA radiation, the intercalated molecule absorbs a photon and can react by C_4-cycloaddition, at either the 3,4 double bond of the pyron ring or the 4′,5′ double bond of the furan ring, with the 5,6 double bond of a pyrimidine base. The furan side monoadduct can still absorb a photon at a wavelength between 300 and 380 nm and, if a pyrimidine base is adjacent to the psoralen monoadduct on the opposite strand, is converted to a diadduct yielding an interstrand cross-link. Linear furocoumarins are bifunctional, being capable of reacting toward the double helix of DNA at both 3,4 and 4′,5′ double bonds. Angelicins, which can only form monoadducts because of their angular structure, are monofunctional.

Thymine is the pyrimidine base mainly involved in photoreaction with furocoumarins. However, the photocycloaddition of some furocoumarin derivatives to cytosine has also been demonstrated.[8,9] More recently, a study on 4′-methyl derivatives of 5-MOP and 5-methoxyangelicin (5-MOA) underlined an unusually high photobinding ability to cytosine in the latter's angular derivatives, whereas the former's linear ones photoreacted with the base thymine, as expected for classic furocoumarins.[10]

The presence of differences in photobinding affinity could reflect different biological consequences. In this context, several studies have been devoted to the interaction of psoralen derivatives with defined DNA fragments.[11,12] In some cases, a possible correlation between the preferential location of DNA damage and the photobiological effects has also been attempted.[13,14]

A quantitative approach to the sequence context effects has also been developed, while trying to obtain kinetics data on the overall photobinding process of various psoralen derivatives to synthetic oligonucleotides.[15] According to this method, an overall rate constant value for the photoreaction of each furocoumarin to a duplex oligonucleotide sequence can be obtained once the equilibrium binding constant is known. Nevertheless, some problems may arise from this approach. It is to be noted that, since the affinity constants are rather low for the complexation of furocoumarins to DNA,[16] the only method that may be suitable for determining them is equilibrium dialysis with radiolabeled compounds. Unfortunately, this is not

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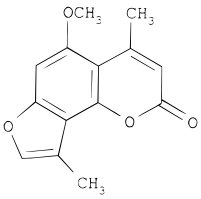
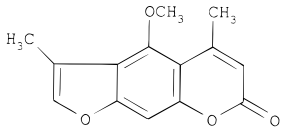


I



II

Figure 1. Chemical structure of test compounds **I** and **II**.

possible using small DNA fragments because of relevant interaction with the cellulose membrane.

Alternatively, some spectroscopic techniques could be employed. However, it should be pointed out that, although the equilibrium binding constant for some furocoumarins can be obtained experimentally, this is not the case for others. Indeed, this is only possible if some physical−chemical properties are satisfied, that is, a remarkable fluorimetric response, suitable spectrophotometric absorption spectra, and finally, a relatively high affinity for the macromolecule. If these conditions are not present, a number of simplifications must be considered.

The aim of this paper is to study the interaction and subsequent photoaddition reaction between furocoumarin derivatives and each active site present in a series of suitable oligonucleotides, without any specific knowledge of the relative binding constants. Two tritium-labeled furocoumarin derivatives were considered: 4,4′-dimethyl-5-methoxyangelicin (compound **I**) because of its unusual photobinding behavior, as highlighted in previous studies[10] and 4,4′-dimethyl-5-methoxypsoralen (compound **II**). The latter derivative is taken as a reference compound because it shows the photoreactive specificity typical of furocoumarin molecules.

These studies were carried out using as substrate 12 different synthetic oligonucleotides, all double-stranded DNA fragments, with known sequence. As these oligonucleotides present a finite number of intercalation sites, we try to correlate the photoaddition of furocoumarin derivatives with the site combination along the double-stranded chain.

In particular, in this report we propose a kinetic approach which allowed us: (a) to correlate the overall rate constant of the photoaddition reaction to the binding and subsequent photoreaction constant of each type of photobinding site present in the oligonucleotide; (b) to identify the type of reactive sites in the double-stranded oligonucleotide chains for furocoumarin derivatives; and (c) to determine how the particular combination of intercalation sites influences reactivity of the sites active in the photoaddition reaction.

Experimental Section

Reagents. 4,4′-Dimethyl-5-methoxyangelicin (compound **I**, Figure 1) and 4,4′-dimethyl-5-methoxypsoralen (compound **II**, Figure 1) were synthesized as described previously.[10] Tritiated **I** and **II** were obtained from Amersham plc (Amersham, Buckinghamshire, U.K.) and purified on thin-layer chromatography plates (silica gel plates Merck art. 5717, 2 mm). Plates were developed with $CHCl_3$. Purified products showed the following specific activities: compound **I**, 1.4×10^7 dpm μmol^{-1}; compound **II**, 2.12×10^7 dpm μmol^{-1}.

O1	5′-TAGCGCTA
O2	5′-CGTTTAAACG
O3	5′-GCGATCGC
O4	5′-CGTATACG
O5	5′-CGTACGTACG
O6	5′-CGTTAACG
O7	5′-TACCCGGGTA
O8	5′-TGCATGCATG GTACGTACGT
O9	5′-TACCGGTA
O10	5′-TACGTACG ATGCATGC
O11	5′-TACGCTAC ATGCGATG
O12	5′-GGTAGGTA CCATCCAT

Figure 2. Oligomer sequences.

Polydeoxyribonucleotides. Poly[dA-dT]·poly[dA-dT] (P-0883, extinction coefficient = 6550 M^{-1} cm^{-1}); poly[dG-dC]·poly[dG-dC] (P-9389, extinction coefficient = 8400 M^{-1} cm^{-1}); poly[dA]·poly[dT] (P-9764, extinction coefficient = 6000 M^{-1} cm^{-1}); and poly[dG]·poly[dC] (P-3136, extinction coefficient = 7400 M^{-1} cm^{-1}) were purchased from Sigma Chemical Co. (St. Louis, MO).

Oligodeoxyribonucleotides. The oligonucleotides were synthesized, at the 1 μmole scale, on an ABI 392 automatic synthesizer (Applied Biosystems, Inc., Foster City, CA), according to cyanoethyl-phosphoramidite chemistry. The average coupling yield was about 98%. Phosphoramidites and reagents for automatic synthesis were from Beckman (Beckman Instruments Inc., Fullerton, CA). The use of Ac-dC phosphoramidite made it possible to perform the cleavage from the solid support and deprotection of the exocyclic amino groups with the AMA reagent (Beckman), 10 min at 65 °C. Each crude oligonucleotide was purified by HPLC on a "Pure DNA" Dynamax column module (C4; 300 A, 5 μm, 21.4 × 50 mm). The terminal 5′-O-dimethoxytrityl protecting group was removed on-column by 0.5% trifluoroacetic acid. The free oligonucleotide was then eluted with a gradient of acetonitrile (3−30%) in triethylammonium acetate (0.1 M, pH 7.0), collected, and lyophilized. The sequence of the oligonucleotides O1−O12 are depicted in Figure 2.

The lyophilized samples of O1−O9 were resuspended in 10 mM TRIS, 0.5 mM EDTA, and 10 mM NaCl, pH = 7.0 (TEN buffer) and placed in a thermostatically controlled water bath at 60 °C (±0.1 °C), which was then allowed to cool to room temperature. To obtain the oligonucleotides O10−O12, the two complementary strands were synthesized, resuspended in TEN buffer, and mixed under gentle stirring. The mixture was then placed in a thermostatically controlled water bath at 60 °C, following the same procedure described above for O1−O9.

The following extinction coefficients per residue were used: oligo O1, 8700 M^{-1} cm^{-1}; O2, 8700 M^{-1} cm^{-1}; O3, 8500 M^{-1} cm^{-1}; O4, 8800 M^{-1} cm^{-1}; O5, 8300 M^{-1} cm^{-1}; O6, 8500 M^{-1} cm^{-1}; O7, 8686 M^{-1} cm^{-1}; O8, 8383 M^{-1} cm^{-1}; O9, 8933 M^{-1} cm^{-1}; O10, 10012 M^{-1} cm^{-1}; O11, 9900 M^{-1} cm^{-1}; O12, 9944 M^{-1} cm^{-1}.

Irradiation Procedures. Small known volumes of concentrated ethanolic solutions of the compounds were added to aqueous solutions (10 mM TRIS, 10 mM NaCl, and 0.5 mM EDTA, pH = 7.0) of each oligonucleotide. The final concentra-

tion of drug was 1.85×10^{-5} M, whereas the oligonucleotide concentration was 4.54×10^{-4} M. The amount of ethanol in the final mixture never exceeded 1%. The mixtures were kept in the dark for 30 min under gentle stirring to allow for the equilibration of the furocoumarin intercalation; then, aliquots (1 mL) of solutions were transferred into a quartz cuvette and irradiated in an Applied Photophysics apparatus with a Xm 300−5 lamp maintained at constant temperature (10 °C ± 0.01) by a refrigerated and heating circulator (Julabo FS18-MV). These conditions are suitable to obtain over 98% of double helix for all oligonucleotides and to overcome the precipitation of furocoumarin in solution. The monochromator wavelength was set at 365 nm, and irradiation power was 1.19 mW/cm^2. After irradiation, each sample was extracted at least five times with a mixture of chloroform/isoamyl alcohol (19:1) for **I** and chloroform/isoamyl alcohol (15:5) for **II** in order to remove all unreacted material and low molecular weight byproducts.

In the experiments on synthetic polynucleotides, buffer solutions of nucleic acids (4.54×10^{-4} M) were added to the labeled compound (1.85×10^{-5} M). Aliquots of these solutions were irradiated for various time periods. Irradiation was carried out using two Philips HPW 125 lamps equipped with a Philips filter emitting over 90% at 365 nm. Radiation intensity was 8.5 mW/cm^2. After irradiation, the solutions were extracted as described above.

Radioactivity Measurements. A known volume (0.1 mL) of the solution was added to 4 mL of Scintillator Emulsifier (Packard, Downers Grove, IL) and counted to evaluate radioactivity due to the presence of labeled compounds. A Packard Model TRI-CARB 4000 liquid scintillation spectrometer was used. The efficiency of the apparatus for counting tritium was within 35−40%.

Linear Programming on the Simplex Method. Linear programming calculations[17−20] were carried out using a Fortran IV program developed in our laboratory. The program is based algorithmically on the implementation of Kuenzi et al.[21] and allows for N independent variables x_i ($1 \leq i \leq 0$) to maximize the function $z = \sum a_i x_i$, which is subjected to the primary constraints $x_1 \geq 0$ and simultaneously to $\alpha = m_1 + m_2 + m_3$ additional constraints. m_1 of these constraints are of the form $\sum a_{1,i} x_i \leq b_i$ with $b_i \geq 0$ and $1 \leq i \leq m_1$. m_2 of these constraints are of the form $\sum a_{2,i} x_i \geq b_j \geq 0$ where $j = m_1 + 1, ..., m_1 + m_2$ and m_3 of them are of the form $\sum a_{k,i} x_i = b_k \geq 0$ with $k = m_1 + m_2 + 1, ..., m_1 + m_2 + m_3$.

Results and Discussion

Photobinding to Synthetic Polynucleotides. It has previously been observed that **I** and **II** show a different pattern in photobinding to nucleic acids having a different base pair composition.[10] In particular, the behavior of linear derivative **II** is similar to that of classical furocoumarins, that is, a linear increase in the amount of drug covalently bound increasing the A−T content. This is due to the fact that thymine is considered by far the preferred pyrimidine base in furocoumarin photobinding. Otherwise, the angular **I** showed unusual behavior because its photoreactivity did not strictly depend on A−T content. Indeed, the study of photobinding to synthetic polynucleotides, poly[dA-dT]·poly[dA-dT] and poly[dG-dC]·poly[dG-dC], showed that the photoreactivity of compound **I** was substantially comparable in both cases, thus indicating a widespread photoaddition capacity along the polynucleotide chain and, therefore, also the ability to react with cytosine.[10]

To appreciate in greater detail the different dependence of the two compounds' photoreactivity on base pair composition,

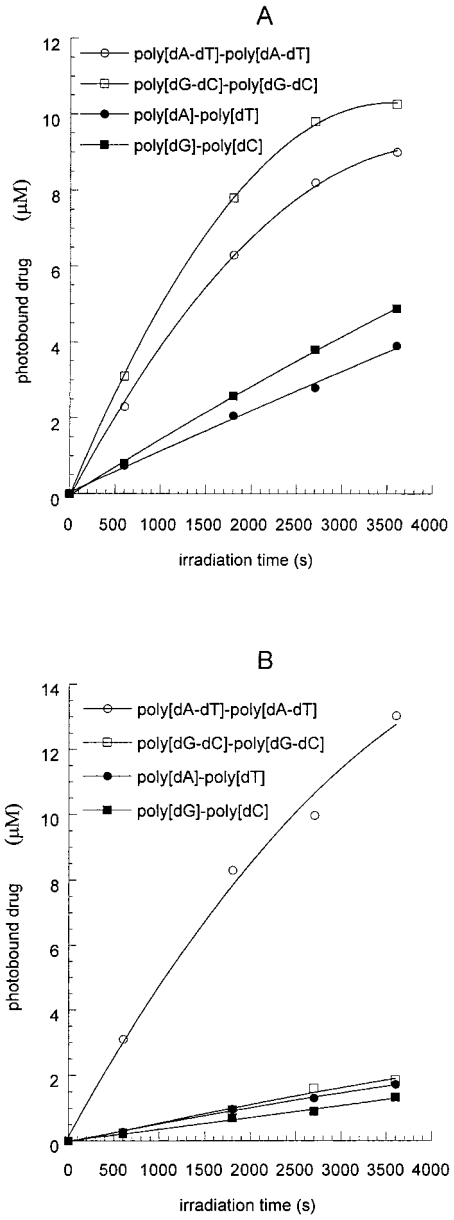


Figure 3. Photobinding of compounds **I** (A) and **II** (B) to synthetic polydeoxyribonucleotides as a function of irradiation time.

we performed a photobinding time course to synthetic polynucleotides having both alternating and consecutive sequences. The results are plotted in Figures 3A and 3B in terms of photobound **I** and **II** (μM) to poly[dA-dT]·poly[dA-dT], poly[dG-dC]·poly[dG-dC], poly[dA]·poly[dT], and poly[dG]·poly[dC], respectively, as a function of irradiation time (s). It appears that the covalent binding ability of compound **I** (Figure 3A) is not strictly related to the particular sequence being considered. In particular, both pyrimidine bases, thymine and cytosine, seem to be recognized equally as targets, whereas only a certain difference arises by considering alternating or consecutive sequences. The results depicted in Figure 3B, corresponding to compound **II**, reveal a very different behavior compared with the previous ones. Indeed, the photobinding of the linear derivative is strongly affected by base pair composition and sequence, being high for alternating A−T and almost nothing for the other types of sequences taken into consideration.

The preferential photobinding to A−T rich sequences is a feature common to nearly all furocoumarins, as well as negligible photoreactivity toward consecutive T or C sites.[14,22,23]

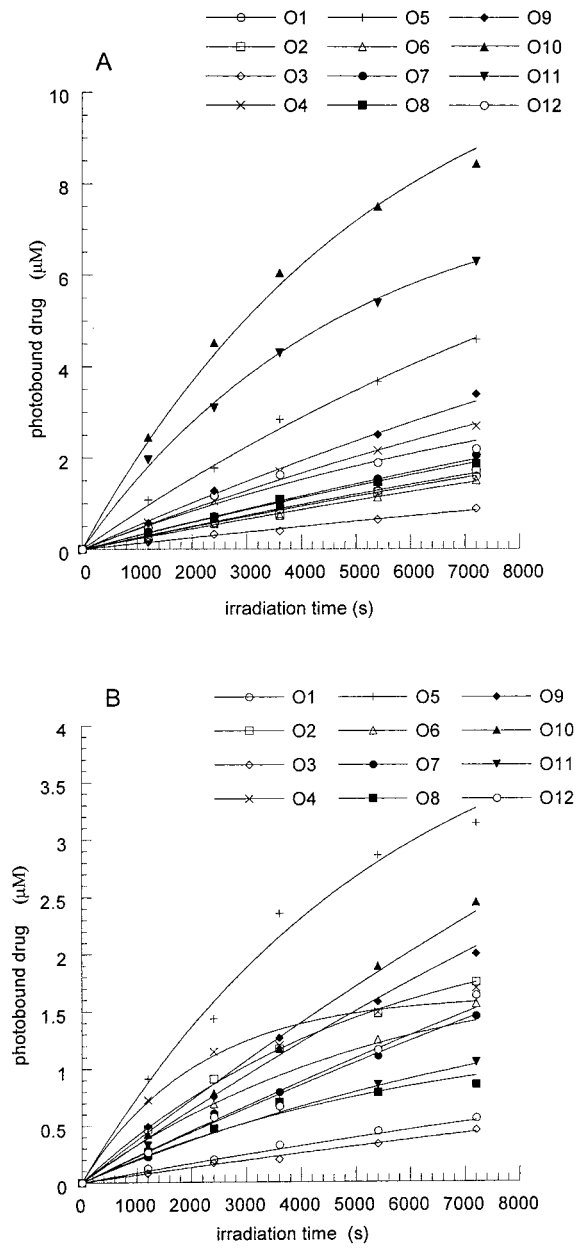


Figure 4. Photobinding of compounds **I** (A) and **II** (B) to the various oligonucleotide sequences. Symbols represent experimental points, and continuous curves are obtained by fitting experimental data by means of eq 8 using the nonlinear least-squares method.

In particular, it has been reported that nonalternating poly[dA]·poly[dT] are not suitable for optimal intercalation and photocycloaddition of various furocoumarins compared with alternating pyrimidine−purine polynucleotides. A difference in local conformation of the double helix may be partly responsible for this behavior.[16,23]

Photobinding to DNA Fragments. To examine the effect due to the sequence context in more depth, we investigated the ability of **I** and **II** to photoadd to twelve synthetic oligonucleotides (called O1−O12), whose sequences are reported in Figure 2.

The results of the covalent photoaddition of test compounds **I** and **II** to the oligomers expressed in terms of photoadduct formed (μM) as a function of irradiation time (s) are reported in Figures 4A and 4B, respectively. Observation of the experimental data allows us to obtain a rough indication of the overall process. It can be noted that the amounts of **I** and **II** bound covalently depend on the particular combination of base sequence, and quite a different trend can be observed in the two cases. In particular, for **I** (Figure 4A) the overall amount of compound photobound is maximum for O10, followed by O11 and O5, whereas for the other oligomers few differences can be detected in the entity of binding. The linear compound **II** (Figure 4B) photoreacts more efficiently with O5.

Furthermore, a striking difference arises from the range of total photoadded amount; this is clearly greater for compound **I** with respect to **II**.

Theoretical Background. Careful examination of the experimental data reported in Figures 4A and 4B seems to indicate that the photoaddition reaction follows first-order kinetics with respect to the disappearance of the binding complex. In previous studies, it has been suggested that the covalent photobinding of furocoumarins to DNA is the result of two subsequent steps. The first is an average equilibrium reaction of binding with the macromolecule, involving aromatic ring stacking, hydrogen bonding, hydrophobic interactions, and van der Waals forces. The second, which requires UVA irradiation, is a covalent photoaddition.[24,25]

Taking this evidence into account, in this paper we try to describe the overall photochemical reaction in terms of single microscopic photoreactions.

In particular, we propose a kinetics model that is based on the following assumptions:

(a) n is the maximum number of binding sites located along the double-stranded polynucleotide;

(b) each site is individualized by a combination of two subsequent base pairs and is singled out by the i-th index;

(c) each type of site, i, is present along the nucleic acid chain with a frequency given by the ratio $f_i = n_i/n$, where n_i is the number of times in which the i-th site appears in a macromolecule that contains n sites;

(d) the overall photoaddition reaction takes place through two consecutive reactions: the first an equilibrium and the second described by a first-order kinetics reaction;

(e) every i-th site in the double-stranded oligonucleotide could potentially photoreact through two consecutive reaction steps. The first is a binding preequilibrium reaction, and the second involves photoaddition of the drug to the nucleic bases. Consequently, each intercalation site is characterized by a binding constant K_i for the intercalation step and a kinetics constant κ_i for the covalent photoaddition process;

(f) according to literature suggestions,[15] the photodegradation reactions are assumed to be negligible.

For every i-th site present along the double-stranded helix we can write the following reaction steps:

$$\begin{aligned} F + S_1 &\overset{K_1}{\rightleftharpoons} FS_1 \xrightarrow{\kappa_1} P_1 \\ F + S_2 &\overset{K_2}{\rightleftharpoons} FS_2 \xrightarrow{\kappa_2} P_2 \\ F + S_i &\overset{K_i}{\rightleftharpoons} FS_i \xrightarrow{\kappa_i} P_i \\ F + S_n &\overset{K_n}{\rightleftharpoons} FS_n \xrightarrow{\kappa_n} P_n \end{aligned} \qquad (1)$$

where F indicates the furocoumarin molecule, S_i the type of i-th site present in the double-stranded nucleic acid, FS_i the complex of intercalation and P_i the corresponding photoaddition product, i-th. K_i and κ_i are the binding constant and kinetics constant, respectively, for the photoaddition of each i-th site along the chain.

Furthermore, the overall reaction rate for the process described by eqs 1 can be written in terms of the disappearance of reagent:

$$-\frac{d[F]}{dt} = \sum_{i=1}^{n} \kappa_i [FS_i] \tag{2}$$

Therefore, if we consider for each site ($1 \le i \le n$) a preequilibrium reaction for the binding intercalation process:

$$K_1 = \frac{[FS_1]}{[F][S_1]} \qquad [FS_1] = K_1[S_1][F]$$

$$K_2 = \frac{[FS_2]}{[F][S_2]} \qquad [FS_2] = K_2[S_2][F]$$

$$K_i = \frac{[FS_i]}{[F][S_i]} \qquad [FS_i] = K_i[S_i][F]$$

$$K_n = \frac{[FS_n]}{[F][S_n]} \qquad [FS_n] = K_n[S_n][F] \tag{3}$$

Substituting eqs 3 into eq 2, we obtain

$$-\frac{d[F]}{dt} = \{\sum_{i=1}^{n} K_i \kappa_i [S_i]\}[F] \tag{4}$$

Moreover, for each type of intercalation site located in the nucleic acid chain the following relationships must be fulfilled:

$$[S_1]^0 = f_1[S]^0 = [S_1] + [FS_1]$$

$$[S_2]^0 = f_2[S]^0 = [S_2] + [FS_2]$$

$$[S_i]^0 = f_i[S]^0 = [S_i] + [FS_i]$$

$$[S_n]^0 = f_n[S]^0 = [S_n] + [FS_n] \tag{5}$$

where $[S_i]^0$ is the initial concentration of i-th type sites, $[S]^0$ is the total concentration of sites, and f_i is the fraction of i-th sites. $[S_i]$ is the concentration at equilibrium of i-th sites, and $[FS_i]$ is the concentration of the i-th complex at equilibrium.

Furthermore, the fractions of sites must satisfy the following condition: $\sum_{i=1}^{n} f_i = 1$.

Substituting eqs 5 into eq 4 and assuming that $[FS_i] \ll [S_i]$ in accordance with the literature,[15] we obtain

$$-\frac{d[F]}{dt} = \{[S]^0 \sum_{i=1}^{n} f_i K_i \kappa_i\}[F] = \xi\,[F] \tag{6}$$

with

$$\xi = [S]^0 \sum_{i=1}^{n} f_i K_i \kappa_i = [S]^0 k_T^{obs}$$

where

$$k_T^{obs} = \sum_{i=1}^{n} f_i K_i \kappa_i = \sum_{i=1}^{n} f_i k_i = \sum_{i=1}^{n} k_i^{obs}$$

$$k_i = K_i \kappa_i \qquad \text{and} \qquad k_i^{obs} = f_i k_i$$

TABLE 1: Kinetics Parameters for the Photoaddition of Compound I to Different Oligonucleotides

oligomer	$[F]_\infty$ (μM)	ξ (s^{-1})	correlation[a] coefficient
O1	7.02 ± 0.0016	$3.6 \times 10^{-5} \pm 1 \times 10^{-7}$	0.99
O2	8.11 ± 0.4	$3.17 \times 10^{-5} \pm 1.6 \times 10^{-6}$	0.99
O3	5.4 ± 0.05	$2.4 \times 10^{-5} \pm 2.3 \times 10^{-7}$	0.99
O4	8.1 ± 0.4	$5.75 \times 10^{-5} \pm 3.2 \times 10^{-6}$	0.99
O5	10.91 ± 0.05	$7.68 \times 10^{-5} \pm 2.4 \times 10^{-6}$	0.99
O6	7.15 ± 0.02	$3.21 \times 10^{-5} \pm 1.2 \times 10^{-7}$	0.99
O7	8.9 ± 0.6	$3.45 \times 10^{-5} \pm 2.7 \times 10^{-7}$	0.99
O8	7.44 ± 0.04	$4.1 \times 10^{-5} \pm 2.82 \times 10^{-7}$	0.99
O9	11.5 ± 0.7	$4.62 \times 10^{-5} \pm 3.5 \times 10^{-6}$	0.99
O10	12.03 ± 1.4	$1.83 \times 10^{-4} \pm 4.1 \times 10^{-5}$	0.98
O11	8.04 ± 0.01	$2.12 \times 10^{-4} \pm 1.5 \times 10^{-6}$	1
O12	4.31 ± 0.32	$1.08 \times 10^{-4} \pm 1 \times 10^{-5}$	0.98

[a] Experimental data were fitted using eq 8 and the NLLS method.[17,20,21,26,27]

The following relation for the concentration of furocoumarin in solution is also valid for each time

$$[F]_0 = [F] + [F]_b \tag{7}$$

where $[F]_0$ is the maximum concentration that can photoreact at time $t = 0$; $[F]_b$ is the concentration that has reacted at time t, and [F] is the concentration of furocoumarin that has not reacted yet. Substituting eq 7 into eq 6 and integrating we obtain

$$[F]_b = [F]_0\{1 - e^{-\xi \cdot t}\} \tag{8}$$

It is evident that for $t \to \infty$, $[F]_b \to [F]_0 \equiv [F]_\infty$, where $[F]_\infty$ is the maximum concentration that can photoreact at time $t \to \infty$.

Equation 8 indicates that the concentration of photoreacted furocoumarin tends to a maximum of saturation at time $t \to \infty$ and to zero for $t \to 0$. The parameter ξ (s^{-1}) is the overall experimental rate constant. This rate constant depends on the sharing of furocoumarin with each type of site that photoreacts along the chain of the nucleic acid (see eq 6). By dividing ξ by $[S]^0$, that is, the total concentration of sites in solution, we obtain the value k_T^{obs} ($M^{-1}\ s^{-1}$), that is, the kinetics constant for the overall event. This latter rate constant shows that photoreaction between the furocoumarin and the nucleic acid depends on: (a) the type of i-th sites present in the nucleic acid; (b) the frequency of appearance of each site along the chain (f_i); (c) the binding constant (K_i) relative to each type of site; and (d) the first-order photoaddition rate constant (κ_i) of each type of site.

Analysis of the Overall Photobinding Process to DNA Fragments. The dependencies on time of the bound furocoumarin concentrations for oligonucleotides depicted in Figure 2 are reported in Figures 4A and 4B for **I** and **II** derivatives, respectively. The experimental data have been fitted by means of eq 8 using the nonlinear least-squares method (NLLS).[17,20,21,26,27] This equation, which describes a pseudo first-order kinetics, allows the overall kinetics parameters to be determined for the photoaddition reaction toward each oligomer. The continuous curves depicted in Figures 4A and 4B are the result of fitting. The fitting parameter values for compounds **I** and **II** are reported in Tables 1 and 2, respectively. $[F]_\infty$ represents the maximum concentration of compound that could photoreact at time $t \to \infty$ and ξ is the overall experimental kinetics constant. In particular, ξ depends on the contribution of each type of site present along the oligomer chain to the overall photoaddition reaction, as indicated by eq 6.

It should be noted that, concerning compound **I** (Table 1), the higher value of ξ was found for oligomers O11 and O10, a slightly lower, even though in the same order of magnitude,

TABLE 2: Kinetics Parameters for the Photoaddition of Compound II to Different Oligonucleotides

oligomer	$[F]_\infty$ (μM)	ξ (s^{-1})	correlation[a] coefficient
O1	$1.94 \pm 5.7 \times 10^{-4}$	$4.81 \times 10^{-5} \pm 1.8 \times 10^{-8}$	0.99
O2	$2.33 \pm 6.13 \times 10^{-3}$	$1.93 \times 10^{-4} \pm 9.7 \times 10^{-6}$	1
O3	$2.78 \pm 1.65 \times 10^{-4}$	$2.40 \times 10^{-5} \pm 3.1 \times 10^{-7}$	0.99
O4	$1.63 \pm 9.8 \times 10^{-3}$	$4.78 \times 10^{-4} \pm 1.1 \times 10^{-5}$	0.98
O5	$4.49 \pm 1.17 \times 10^{-2}$	$1.807 \times 10^{-4} \pm 2.1 \times 10^{-5}$	0.99
O6	$1.90 \pm 1.47 \times 10^{-2}$	$1.89 \times 10^{-4} \pm 6.9 \times 10^{-7}$	0.97
O7	$6.44 \pm 1 \times 10^{-3}$	$3.57 \times 10^{-5} \pm 5.8 \times 10^{-9}$	0.99
O8	$1.14 \pm 4.4 \times 10^{-3}$	$2.17 \times 10^{-4} \pm 1.7 \times 10^{-6}$	0.98
O9	7.33 ± 0.35	$4.58 \times 10^{-5} \pm 2 \times 10^{-6}$	0.99
O10	9.36 ± 0.23	$4.05 \times 10^{-5} \pm 1.1 \times 10^{-6}$	0.99
O11	$2.73 \pm 1.44 \times 10^{-3}$	$6.96 \times 10^{-5} \pm 1 \times 10^{-7}$	0.99
O12	8.05 ± 0.8	$2.92 \times 10^{-5} \pm 3.3 \times 10^{-6}$	0.98

[a] Experimental data were fitted using eq 8 and the NLLS method.[17,20,21,26,27]

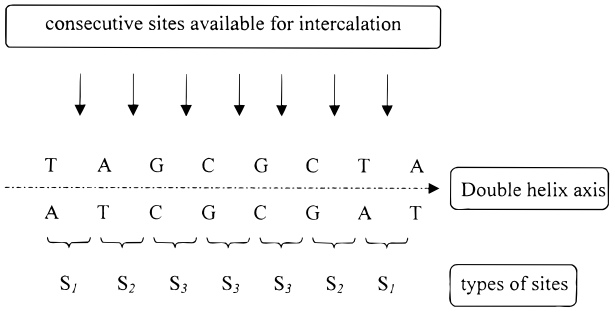


Figure 5. Schematic representation of intercalation sites present in the double helix sequence of oligomer O1. Each type of site S_i is singled out by a combination of two consecutive base pairs along the helix axis.

value was calculated for O12. For all other oligomers, the ξ value is significantly lower and decreases in the following order: O5 > O4 > O9 > O8 > O1 > O7 > O6 > O2 > O3.

The kinetics parameters calculated for derivative **II** (Table 2) show a different pattern with respect to those of the angular isomer; indeed, the higher value of ξ corresponds to the photoreaction with oligomer O4, and this value is about twice that of the subsequent one, for the oligomer O8. For other fragments, the following reactivity order is detected: O2 > O6 > O5 > O11 > O1 > O9 > O10 > O7 > O12 > O3.

Kinetics Analysis of Site Reactivity. An important goal in the study of reactivity of **I** and **II** toward different oligonucleotides of known sequence is to determine the reactivities associated with each single site present along the oligonucleotide chain from the measurable overall reactivities ξ. This is a crucial point in the photoreaction of furocoumarins to the DNA macromolecule. Indeed, if the properties of different reaction sites present along a known oligonucleotide are determined, we can study if these properties are (a) transferable to other oligonucleotides having a different sequence and (b) influenced by the site combination along the oligonucleotide chains. For this purpose, we used oligonucleotides with a known sequence to identify the type of different sites and the frequency of their appearance along the chains. In Figure 5, the possible intercalation sites present in the oligomer O1 are reported by way of an example. Considering that the intercalation of the furocoumarin molecule inside the double helix involves four bases, that is, two consecutive base pairs, it appears evident that because the furocoumarin can freely rotate in solution before intercalation takes place, each type of site can be represented by more than one configuration of the same combination of four bases, which gives equivalent sites from the reactivity point of view. Thus, starting with the sequences of DNA fragments reported in Figure 2, we singled out six different types of sites (named S_x, where $1 \leq x \leq 6$). In Table 3, all six site combinations and their possible configurations along the oligonucleotide chains are summarized.

TABLE 3: Base Pair Combinations Individualizing All Possible Intercalation Sites Present in O1−O12 Oligomers

site	base pair combinations			
S_1	TA	AT		
	AT	TA		
S_2	AG	GA	TC	CT
	TC	CT	AG	GA
S_3	GC	CG		
	CG	GC		
S_4	TT	AA		
	AA	TT		
S_5	GT	TG	CA	AC
	CA	AC	GT	TG
S_6	CC	GG		
	GG	CC		

TABLE 4: Frequency of Appearance (f_i) of the Six Types of Possible Intercalation Sites for Each Oligomer[a]

oligomer	f_1	f_2	f_3	f_4	f_5	f_6
O1	2/7	2/7	3/7	-	-	-
O2	1/9	-	2/9	4/9	2/9	-
O3	1/7	2/7	4/7	-	-	-
O4	3/7	-	2/7	-	2/7	-
O5	2/9	-	3/9	-	4/9	-
O6	1/7	-	2/7	2/7	2/7	-
O7	2/9	-	1/9	-	2/9	4/9
O8	1/7	-	2/7	-	4/7	-
O9	2/7	-	1/7	-	2/7	2/7
O10	2/7	-	2/7	-	3/7	-
O11	2/7	1/7	2/7	-	2/7	-
O12	2/7	1/7	-	-	2/7	2/7

[a] $f_i = n_i/n$, where n_i is the number of times the i-th site is detected along the chain and n is the total number of sites contained within the oligomer.

As mentioned above, the frequency of appearance of a site type is defined as $f_i = n_i/n$, where n_i is the number of times the i-th site is detected along the chain and n is the total number of sites contained inside an oligomer. This number can easily be calculated if we take into account that the number of potential intercalation sites is $N - 1$ for a chain with N base pairs. Table 4 reports the f_i values for each oligonucleotide.

According to eq 6, by dividing the values of ξ reported in Tables 1 and 2 by $[S]^0$, we obtain the corresponding values of k_T^{obs}. As previously reported, for each oligomer k_T^{obs} is given by

$$k_T^{obs} = \sum_{i=1}^{n} f_i k_i \qquad (9)$$

where $k_i = K_i \kappa_i$ is the overall kinetics constant corresponding to the i-th site, and f_i is its frequency of appearance along the oligomer chain.

It is clear from eq 9 that k_T^{obs} is the sum of all events involved in the photocombination process multiplied by their frequency of appearance. Therefore, at first the numerical solution of systems of eqs 9 for oligomers having the same intercalation sites seems to be the simplest way of determining k_i for each site. Given that the values of k_i which give rise to k_T^{obs} are all physical quantities that are positive or zero, the method of system equation solution becomes inapplicable. Consequently, it is possible to single out their own contribution to k_T^{obs} by using a suitable program of calculations based on the linear programming and simplex method (LPSM).[17,19−21] This

TABLE 5: Kinetics Constants of the Overall Photoaddition Process (k_T^{obs}) and Kinetics Constants Relative to Each Binding Site (k_i)[a] for Compound I

oligomer	k_T^{obs} (M^{-1} s^{-1})	k_1 (M^{-1} s^{-1})	k_2 (M^{-1} s^{-1})	k_3 (M^{-1} s^{-1})	k_4 (M^{-1} s^{-1})	k_5 (M^{-1} s^{-1})	k_6 (M^{-1} s^{-1})	b
O1[c]	0.0793	0.2223	-	0.0368				α
O2	0.0698	0.3618		-	0.0666	-		β
O3	0.0528	0.2223	-	0.0368				α
O4	0.1266	0.2281		-		0.1010		δ
O5	0.1692	0.2281		-		0.1010		δ
O6	0.0707	0.3618		-	0.0666	-		β
O7	0.076	-		0.7053		-	0.0009	γ
O8	0.0903	0.2281		-		0.1010		δ
O9	0.101	-		0.7053		-	0.0009	γ
O10	0.4031	-		1.4109		-		ϵ
O11	0.4669	-	0.4467	1.4109		-		ϵ
O12	0.2378	-	0.4467			-	0.6090	ϵ

[a] k_i values were obtained by means of a Fortran IV program based on the LPSM method (Linear Programming and the Simplex Method).[17,19−21] [b] Symbols refer to the five LPSM compatibility classes. [c] - indicates the sites present in the oligomer that are not involved in the photoaddition process.

TABLE 6: Kinetics Constants of the Overall Photoaddition Process (k_T^{obs}) and Kinetics Constants Relative to Each Binding Site (k_i)[a] for Compound II

oligomer	k_T^{obs} (M^{-1} s^{-1})	k_1 (M^{-1} s^{-1})	k_2 (M^{-1} s^{-1})	k_3 (M^{-1} s^{-1})	k_4 (M^{-1} s^{-1})	k_5 (M^{-1} s^{-1})	k_6 (M^{-1} s^{-1})	b
O1[c]	0.105	0.3653	-	0.0014				α
O2	0.4251	2.0022		-	0.4560	-		β
O3	0.053	0.3653	-	0.0014				α
O4	1.053	0.2361		-		0.7775		δ
O5	0.3980	0.2361		-		0.7775		δ
O6	0.4162	2.0022		-	0.4560	-		β
O7	0.0788	-		0.7053		-	0.0009	γ
O8	0.478	0.2361		-		0.7775		δ
O9	0.101	-		0.7053		-	0.0009	γ
O10	0.0892	-		0.3122		-		ϵ
O11	0.1533	-	0.4488	0.3122		-		ϵ
O12	0.0643	-	0.4488			-	0.0007	ϵ

[a] k_i values were obtained by means of a Fortran IV program based on the LPSM method (Linear Programming and the Simplex Method).[17,19−21] [b] Symbols refer to the five LPSM compatibility classes. [c] - indicates the sites present in the oligomer that are not involved in the photoaddition process.

method is most suitable to solving the systems of eqs 9 because (a) "non-negativity" is a reasonable necessary constraint on any variable k_i; (b) we treat a process of linearly additive limitations imposed by the experiment; and (c) the functions that we must optimize are linear.

On the other hand, this analysis allows us to classify the oligomers into different groups. These groups are characterized by the compatibility of eqs 9 for the resolution of the particular system.[17,21] Therefore, this method was applied to the determination of site photoaddition reactivity.

Kinetics Parameters Belonging to Each Site of Photoaddition. The values of k_T^{obs} (M^{-1} s^{-1}), obtained as described previously, are shown in Tables 5 and 6 for compounds **I** and **II**, respectively. In the same tables, the values of k_i ($1 \leq i \leq 6$) determined by linear programming optimization and relative to each *i*-th binding site for all oligomers are also reported. The five compatibility classes individualized from the analysis performed using the LPSM method[17,19−21] are indicated by Greek letters and are in the last column of Tables 5 and 6. A compatibility class is a group of oligomers having the same type of intercalation site and whose system of eq 9 is resolvable by linear programming optimization. This analysis also shows that some types of sites do not contribute appreciably to the overall kinetics process, even if they are present inside the oligomer.

Considering the angular compound **I** (Table 5), the O11 and O10 fragments exert higher values of k_T^{obs}, followed by O12. These three oligomers belong to the same compatibility class, ϵ, and the calculated k_i values corresponding to the sites S_2, S_3, and S_6, show a very interesting pattern. In particular k_3 indicates that site S_3, characterized by the alternating G−C base pair, when present, appears to be kinetically the most relevant. Site S_6, containing consecutive C or G sequences, also shows a significantly high k_6 value. The lower value is that found for S_2, which contains both thymine and cytosine alternating along the same chain. By comparing the k_i values for S_2 and S_5 sites, both present in O11 and O12, the only preference toward site S_2 can be noted. This means that the C_4-cycloaddition process, involving the 5,6 double bond of the pyrimidine base, is kinetically promoted by the presence of the other pyrimidine base in the same direction along the double helix. Furthermore, site S_1, showing alternating A−T sequences, although considered by far the preferred target in psoralen photobinding, does not contribute to the overall kinetics process of compound **I** toward these DNA fragments. These observations confirm the above-mentioned remarks regarding the unusual behavior of photobinding of this compound, which exerts a good ability to photoreact with cytosine.

It is worth mentioning that this approach also makes it possible to assess if the photoreactivity of a certain site is dependent or not on the base composition of its flanking sites. The comparison between values of k_3 calculated for three different classes of compatibility, that is, α (O1 and O3), γ (O7 and O9), and ϵ (O10, O11 and O12), reveals that this kinetics constant strongly depends on surroundings. Indeed, it appears that the photoreaction is kinetically more favorable if site S_3 belongs to nonpalindromic DNA fragments (O10 and O11). Of palindromic DNA fragments, the k_3 values are higher when the corresponding site (S_3) is surrounded by cytosine or guanine bases (O7 and O9) instead of adenine or thymine (O1 and O3).

A completely different pattern emerges when observing the values of k_1 calculated for the sites belonging to the compat-

ibility classes α, β, and δ. Indeed, its value remains practically constant, thus indicating that the kinetics constant for the photoaddition of compound **I** toward site S_1 does not depend on the position of the site along the sequence.

Analysis of the kinetics parameters relative to the photoaddition of linear compound **II** to oligomers O1−O12 (Table 6) enables us to examine in more depth the previously underlined differences in photoreaction ability with respect to angular derivative **I**. The higher k_T^{obs} is relative to oligomer O4, thus indicating that it contains the most photoreactive sites in the most favorable conditions for photoaddition. Kinetics analysis reveals that for this oligomer, S_5 ($k_5 = 0.7775\ M^{-1}\ s^{-1}$) makes a greater contribution to photoaddition. Site S_1 also contributes, even if to a lesser extent ($k_1 = 0.2361\ M^{-1}\ s^{-1}$). O5 and O8 belong to the same compatibility class of O4 (δ), and their k_T^{obs} values are almost half compared with that of O4. The k_T^{obs} values of O2 and O6, belonging to another compatibility class, β, also show the same order of magnitude as those of O5 and O8. The k_T^{obs} values relative to the photoreaction of compound **II** toward all other oligomers are significantly lower.

Regarding the δ compatibility class, k_1 and k_5 are the components accountable for the k_T^{obs} value for the photoreaction of **II** with oligomers O4, O5, and O8. It can be observed that k_3 plays no role in the overall kinetics process, thus indicating that site S_3 is prohibited for the covalent photoaddition of linear compound **II** to these DNA fragments. A previous work underlined a high reactivity of linear furocoumarin 5-MOP to GTAC-containing sequences.[15] This sequence is constituted by two subsequent S_5 sites (Table 3); therefore the behavior of compound **II** seems to reflect the classic preference exerted by psoralens in the photoaddition reaction. Taking into consideration O2 and O6, of the β compatibility class, it is interesting to note that they are characterized by consecutive T or A sequences. In this case, S_1 is by far the preferred site, followed by S_4. Both sites are characterized by the concurrent presence of the bases thymine and adenine, on the same strand in S_1 or on the opposite strand in S_4 (see Table 3). Sites S_3 and S_5, where cytosine and guanine are present, do not contribute to the overall kinetics process, although they are present in the oligomer sequence. This trend reflects that of the classical furocoumarins, for which A−T rich sequences are the preferential site for both intercalation and photoreaction.[22]

Comparing k_1 values of distinct compatibility classes, it is possible to observe that they are appreciably different. This means that the photoreactivity of the S_1 site depends on the surroundings. Comparing k_1 values reported in Table 6 with oligomer sequences depicted in Figure 2, it appears that adjacent runs of T_n/A_n constitute preferential flanking sequences (see O2 and O6).

Conclusions

In this paper, the photobinding reaction of angular (**I**) and linear (**II**) furocoumarin compounds toward twelve oligonucleotides was studied. It was assumed that this reaction takes place over two consequent reaction steps. The first step is a preequilibrium reaction, and the second is a first-order kinetics reaction. This approach, applied to the experimental binding data, allows us to evaluate the overall photoreaction kinetics constant. In the sequences studied, the use of well-characterized oligonucleotides allows six possible intercalation sites to be individuated and their frequency (f_i) to be determined for each oligonucleotide chain. These frequencies, the overall photoreaction kinetics constant, and eq 9 permitted us to determine, by linear programming optimization, the overall photoreactive kinetics constants of each photoreactive intercalation site, k_i.

Considering the approaches together, as well as allowing an accurate determination of the kinetics parameters, it is possible to resolve the overall covalent photoaddition process into the single kinetics events that occur at the level of base pairs and highlight several differences in kinetics behavior for the two furocoumarin derivatives analyzed. This analysis strongly indicates that the reactivity of the base pair sequences depends on (a) the type of base pairs, (b) their sequence, and (c) their surroundings within the chains.

The studies devoted to identifying the specific sites involved in the interaction with DNA are aimed at obtaining phototherapy drugs that are more efficient, better targeted, and without undesired side effects. In this perspective, this new methodological approach may be a valuable tool for investigations into the relationship between the photobiological effects evoked by phototherapeutic drugs and the molecular target.

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