Synthesis and Characterization of New Psoralen Derivatives with Superior Photoreactivity with DNA and RNA†

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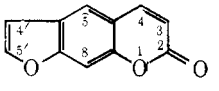
ABSTRACT: The synthesis of five new psoralen derivatives is described. Three of these, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen, 4′-methoxymethyl-4,5′,8-trimethylpsoralen, and 4′-aminomethyl-4,5′,8-trimethylpsoralen hydrochloride, are characterized with respect to their photoreactivity with DNA and RNA. They are found to be greatly superior to 4,5′,8-trimethylpsoralen and 8-methoxypsoralen, the two commonly used psoralens, in their abilities to saturate the photoreactive sites on DNA and RNA without repeated addition of reagent. A simplified mechanism for the photoreaction of psoralens with nucleic acids is presented and provides a basis for understanding the superior properties of these compounds. The compounds have superior reactivity not only with isolated DNA and RNA but also in viruses and in cells. Psoralens are shown for the first time to cross-link RNA double helices.

The linear isomers of the furocoumarin family known as psoralens were first recognized as potent dermal photosensitizing agents by the ancient Egyptians who employed fruit and seed extracts containing them for centuries in the treatment of vitiligo (Perone, 1972). More recently, psoralens have drawn attention as photochemotherapeutic agents used in the treatment of psoriasis (Parrish et al., 1974).

Psoralens photoreact with nucleic acids when irradiated with long-wavelength ultraviolet light (365 nm). The products include mono- and diadducts to pyrimidine bases (Musajo et al., 1967). Diaddition results in a covalent interstrand bridge or cross-link (Cole, 1970, 1971). It is apparent there are several steps involved in cross-link formation in nucleic acid double helices. The probability of cross-link formation may be represented as the product of three independent probabilities: (1) the probability of intercalative binding to the helix, (2) the probability of monoaddition once intercalation has occurred, and (3) the probability of diaddition conditioned upon monoaddition having occurred. Each of these probabilities may be investigated as a function of the type and position of the substituents on the parent psoralen nucleus (**1**). The two most



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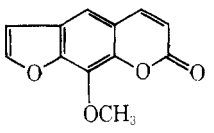

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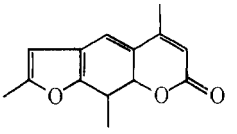

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widely used psoralen derivatives are 8-methoxypsoralen (**2**, methoxsalen[1]) and 4,5′,8-trimethylpsoralen (**3**, trioxsalen). In this report, we present five new psoralen derivatives, three of which we have found to be useful nucleic acid reagents.

The objective of the syntheses described here is the preparation of psoralen derivatives which are superior to the known psoralens with respect to monoaddition to nucleic acid. Superior, in this sense, refers to the resulting density of adduct on the nucleic acid without replenishment of reagent at a common initial concentration of binding sites and total psoralen concentration. The formation of cross-link is of interest only as a secondary consequence of high efficiency in monoaddition. We have ignored questions of photoefficiency or quantum yield in the design of these derivatives because such questions are deemed of little importance in the early stages of this study, since adjustments of excitation intensity are experimentally accessible.

We have synthesized compounds with two advantages: (1) they have increased solubility in water, and (2) they have low dissociation constants from nucleic acids. We have started these syntheses with trioxsalen (4,5′,8-trimethylpsoralen) in order to take advantage of the low dissociation constant of this derivative in its binding to DNA relative to the other commercially available psoralens.

A more extensive synthetic project is also in progress in which similar halo, hydroxy, methoxy, and amino-alkyl derivatives with substitutions at positions other than the 4′ position of psoralen are being made.

Materials and Methods

Syntheses of New Psoralen Derivatives. 4′-Chloromethyl-4,5′,8-trimethylpsoralen (**4**). Trioxsalen (**1**, 659 mg, 2.89 mmol) was dissolved in 75 mL of glacial acetic acid by gentle heating and cooled to room temperature, 5 mL of chloromethyl methyl ether was then added, and the mixture was allowed to sit at room temperature for 24 h, followed by a second 5 mL addition of the ether. After 48 h, the reaction flask was placed on ice and 12 h later an abundant white precipitate was collected (435 mg). Another crop was isolated from the filtrate to give 499 mg of total product (62.5%): mp 215–217 °C; NMR ($CDCl_3$) δ 2.6–2.7 (9 H, m, C4,5′,8-methyls), 4.8 (2 H, s, CH_2Cl), 6.3 (1 H, s, C3-H), 7.6 (1 H, s, C5-H); mass spectrum *m/e* (relative intensity) 241 (100), 276 (M^+, 48), 278 (M + 2, 15).

Anal. Calcd for $C_{15}H_{13}ClO_3$: C, 65.1; H, 4.7; Cl, 12.8. Found: C, 65.0; H, 4.8; Cl, 12.6.

4′-Hydroxymethyl-4,5′,8-trimethylpsoralen (**5**). 4′-Chloromethyl-4-5′,8-trimethylpsoralen (**4**, 53 mg, 0.19 mmol) in

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[1] Abbreviations used are: trioxsalen, 4,5′,8-trimethylpsoralen; methoxsalen, 8-methoxypsoralen; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

50 mL of distilled water was refluxed for 3 h, followed by cooling on ice for 2 h. The product was removed by filtration giving 25 mg (50.5%): mp 221–224 °C; NMR (Me_2SO-d_6) δ 2.5–2.7 (9 H, m, C4,5′,8-methyls), 4.5–4.7 (2 H, s, CH_2OH), 5.0–5.2 (1 H, bs, CH_2OH), 6.3 (1 H, s, C3-H), 7.8 (1 H, s, C5-H); mass spectrum *m/e* (relative intensity) 241 (17), 258 (M^+, 100).

Anal. Calcd for $C_{15}H_{14}O_4$: C, 69.8; H, 5.4. Found: C, 69.5; H, 5.5.

4′-Methoxymethyl-4,5′,8-trimethylpsoralen (**6**). 4′-Chloromethyl-4,5′,8-trimethylpsoralen (**4**, 78 mg, 0.28 mmol) was refluxed in 30 mL of methanol for 2 h. Evaporation of the solvent gave 74 mg (97.2%) of product, which was recrystallized from methanol: mp 171–174 °C; NMR ($CDCl_3$) δ 2.4–2.6 (9 H, m, C4,5′, 8-methyls), 3.4 (3 H, s, CH_2OCH_3), 4.6 (2 H, s, CH_2OCH_3), 6.3 (1 H, s, C3-H), 7.8 (1 H, s, C5-H); mass spectrum *m/e* (relative intensity) 241 (100), 272 (M^+, 93).

Anal. Calcd for $C_{16}H_{16}O_4$: C, 70.6; H, 5.9. Found: C, 70.4; H, 5.9.

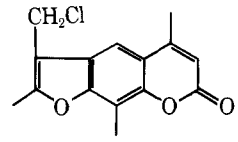
4′-N-Phthalimidomethyl-4,5′,8-trimethylpsoralen (**7**). 4′-Chloromethyl-4,5′,8-trimethylpsoralen (**4**, 200 mg, 0.73 mmol), potassium phthalimide (165 mg, 0.89 mmol, purified by digestion at reflux 2 h in acetone followed by vacuum drying, 100 °C at 1 torr for 6 h (Gilman, 1941)) and 20 mL of *N,N′*-dimethylformamide were heated at 100 °C for 6 h with constant stirring. The solvent was evaporated in vacuo by heating in a water bath, leaving behind a yellow paste which was taken up in chloroform and washed three times with water. The chloroform was dried over $MgSO_4$ and then filtered and evaporated to give 222 mg (79.3%) of product: mp 267–274 °C; NMR ($CDCl_3$) δ 2.5–2.8 (9 H, m, C4,5′, 8 methyls), 5.0 (2 H, s, CH_2N), 6.3 (1 H, s, C3-H), 7.7–7.8 (4 H, d, phthal), 8.0 (1 H, s, C5-H); mass spectrum *m/e* (relative intensity) 240 (75), 241 (20), 387 (M^+, 80).

4′-Aminomethyl-4,5′,8-trimethylpsoralen Hydrochloride (**8**). 4′-*N*-Phthalimidomethyl-4,5′,8-trimethylpsoralen (**8**, 848 mg, 2.2 mM), hydrazine hydrate (85% in water, 0.5 mL), and 95% ethanol (100 mL) were refluxed for 4 h, followed by a second 0.5-mL addition of the hydrazine hydrate solution. After extending the reflux 2 h, no starting material remained, as determined by TLC (diethyl ether). The ethanol was evaporated and the residue was taken up in 200 mL of 0.1 N NaOH, followed by extraction with three 50-mL portions of chloroform to give 193 mg (34%) of the crude amine. To prepare the hydrochloride, the amine was taken up in 100 mL of 1.2 N HCl, which was extracted with three 30-mL portions of chloroform to remove impurities. Evaporation in vacuo of the acidic solution gave the crude hydrochloride, which was dissolved in enough absolute ETOH and precipitated by the addition of an equal volume of diethyl ether. After cooling overnight (7 °C), 161 mg of pure product was collected: mp 260–269 °C; NMR ($CDCl_3$) as the amine δ 1.4–1.6 (2 H, bs, CH_2NH_2), 2.6–2.7 (9 H, m, C4,5′, 8 methyls), 4.1 (2 H, s, CH_2NH_2), 6.3 (1 H, s, C3-H), 7.7 (1 H, s, C5-H); mass spectrum *m/e* (relative abundance) 240 (100), 257 (M^+, 36).

Anal. Calcd for $C_{15}H_{16}ClNO_3$: C, 61.3; H, 5.5; N, 4.8; Cl, 12.1. Found: C, 61.0; H, 5.5; N, 4.7; Cl, 11.9.

Synthesis of Tritiated Derivatives. 4,5′,8-[^{3}H]Trimethylpsoralen. 4,5′,8-Trimethylpsoralen (1153 mg), T_2O)aqueous, 100 Ci in 4 mL), dioxane (67.5 mL), and fuming H_2SO_4 (30% SO_3, 7.5 mL) were refluxed for 2 h with constant stirring, followed by cooling to room temperature, addition of 125 mL of ice water, and cooling on ice for 1 h. The precipitate was collected by filtration and air dried to give 900 mg (78%) of crude product; mass spectrum *m/e* (relative abundance) 228 (M^+, 100). A small amount of the material (30 mg) was dissolved in 75 mL of absolute ethanol, 2 g of nuchar was added, and the mixture was refluxed for 10 min and then immediately filtered (hot) through a fine-sintered-glass filter to remove the charcoal. The filtrate was evaporated and the residue was recrystallized from methanol–water (90:10). Analytical TLC of the product ($CHCl_3$–CH_3OH, 98:2) showed >95% of the counts in the trimethylpsoralen. The specific activity of the compound was determined by counting aliquots of an absolute ethanol solution of known concentration in toluene–omnifluor and found to be 1.7×10^6 cpm/μg.

Other Derivatives. The tritiated new psoralen derivatives (**4–8**) were prepared as described above starting with the tritiated 4,5′,8-trimethylpsoralen.



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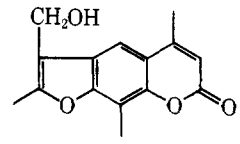

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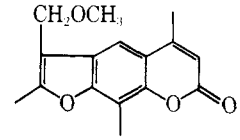

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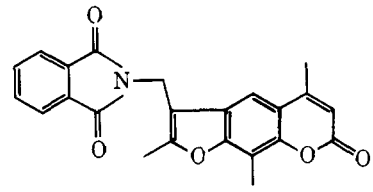

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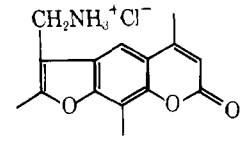

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Dark (Noncovalent) Binding of the Psoralen Derivatives to Nucleic Acid. Calf-thymus DNA (Sigma type I) was dissolved in 0.01 M Tris, 0.001 M EDTA, pH 8.5, buffer at a concentration of 25 μg/mL; 1 mL of this DNA solution was placed in a dialysis bag (pretreated by boiling in $NaHCO_3$); and the various tritiated derivatives were added inside the bag in half the cases and outside the bag in the other half. The molar ratio of psoralen molecules to base pairs was approximately 1:25. The bags were placed in vials filled with 18 mL of buffer and put on a shaker for 48–60 h. After this period, radioactivity was determined both inside and outside the bags and the optical density of the DNA solution was measured. From this information and the specific activity of each derivative, the amount of drug bound to the DNA was determined. Binding of the derivatives to *Drosophila melanogaster* ribosomal RNA was measured in exactly the same manner.

Light (Covalent) Binding of Psoralen Derivatives to Nucleic Acid. DNA and RNA used in the light-binding experiments have been described in the previous section. Samples (5 mL) of each nucleic acid were prepared at a concentration of 25 μg/mL in 0.01 M Tris, 0.001 M EDTA buffer; the radioactive psoralen derivatives were added in a ratio of one psoralen for every three base pairs; and the irradiation was carried out in one of the two following devices.

The low-intensity irradiations were performed with a modified slide projector, which was fitted with a 400-W General Electric mercury-vapor lamp (H 400 A 33-1/T16).

TABLE I: Extinction Coefficients, Solubilities, Dissociation Constants, and Ratios of Concentrations of Occupied to Unoccupied Binding Sites in Saturated Solutions for Psoralen Derivatives.[a]

Compounds	ϵ_{250nm} (L mol^{-1} cm^{-1}) (1)	Solubility μg/mL (2)	Solubility mol/L (3)	K_D DNA mol/L (4)	Column 3/ Column 4 [PS]/[S] for DNA in Saturated Solution (5)	K_D RNA mol/L (6)	Column 3/ Column 6 [PS]/[S] for RNA in Saturated Solution (7)
8-Methoxypsoralen	1.9×10^4	36	1.7×10^{-4}	2.5×10^{-3}	0.068	1.7×10^{-2}	0.010
4,5′,8-Trimethylpsoralen	1.8×10^4	0.6	2.6×10^{-6}	5.6×10^{-5}	0.046	$\sim 10^{-4}$	0.026
4′-Methoxymethyl-4,5′,8-trimethylpsoralen	2.1×10^4	10	3.7×10^{-5}	9.4×10^{-5}	0.39	$\sim 10^{-3}$	0.037
4′-Hydroxymethyl-4,5′,8-trimethylpsoralen	2.5×10^4	41	1.6×10^{-4}	2.9×10^{-4}	0.55	$\sim 10^{-3}$	0.16
4′-Aminomethyl-4,5′,8-trimethylpsoralen hydrochloride	2.5×10^4	10^4	3.4×10^{-2}	6.6×10^{-6}	5000	2×10^{-5}	1700

[a] The data in Table I for 8-methoxypsoralen in columns two through seven were calculated from results presented by Dall'acqua and Rodighiero (1966). The remaining measurements were performed by the authors.

The image of the arc was focused on the same cell, jacketed by a cobaltous nitrate solution which was also used for the high-intensity irradiations. The light intensity delivered to the sample in this device was 4 to 6 mW/cm^2.[2] The high-intensity irradiations were carried out in a device containing two of the same 400-W General Electric mercury-vapor lamps, which were mounted on either side of a double-walled sample chamber at a distance between centers of 4.0 cm. The chamber was cooled to 10 °C by continuous circulation of a temperature regulated solution of cobaltous nitrate (40%, w/w). The cobalt solution served as an ultraviolet filter, which allowed a maximum transmittance of 365-nm light and a window from approximately 340–380 nm. The intensity of the light at the surface of the inner sample chamber was approximately 100 mW/cm^2. The nucleic acid–psoralen mixture was placed in the inner chamber where it was continuously stirred by a small magnetic bar throughout the irradiation, and 1-mL aliquots of each solution (derivative plus nucleic acid) were taken at 20, 40, and 60 min. Each aliquot was then extracted twice with an equal volume of chloroform–isoamyl alcohol (24:1) to remove unreacted psoralen, followed by exhaustive dialysis against 0.01 M Tris, 0.001 M EDTA buffer. We observed that successful extraction of unbound 4,5′,8-trimethylpsoralen and its photobreakdown products by the chloroform–isoamyl alcohol required the aqueous phase to be at least 0.15 M in NaCl. Much of the 4′-aminomethyl-4,5′,8-trimethylpsoralen remains in the aqueous phase and is removed by dialysis. Finally, the optical density of the nucleic acid–psoralen mixture was taken and its radioactivity was determined, thus giving the amount of derivative covalently bound to the DNA or RNA.

Electron Microscopy. Photo-cross-linked DNA and RNA were denatured in a solution containing 0.5 M glyoxal (Cech and Pardue, 1976), 70% formamide, 0.01 M Na_2HPO_4 buffer, pH 7.5, 0.001 M EDTA at 37 °C for 30 to 60 min. The hyperphase contained 50% formamide, 0.1 M Tris, 0.01 M EDTA, pH 8.4, and 40 to 50 μg/mL of cytochrome *c*, while the hypophase is 0.01 M Tris, 0.001 M EDTA, pH 8.4, and 17% formamide resulting in isodenaturing conditions (Davis et al., 1971).

Cross-links were identified on electron micrographs by the presence of single-strand loops in the DNA and RNA and from the double-strand appearance of the molecules under these denaturing conditions (Hanson et al., 1976).

Results

Properties of the New Psoralen Derivatives. The dissociation constant for the noncovalent binding of each derivative to nucleic acid is defined by the expression

$$K_D = \frac{[P][S]}{[PS]}$$

where [P] is the concentration of free psoralen, [S] is the concentration of unoccupied binding sites where each base pair is considered to be a binding site, and [PS] is the concentration of bound sites.

The results of the equilibrium dialysis measurements are presented in Table I. The units of the dissociation constants are moles/liter. Solubilities and molar extinction coefficients were obtained in pure water, and the equilibrium constants were obtained in 0.01 M Tris–0.001 M EDTA, at pH 8.4.

Covalent Binding of Psoralen Derivatives to DNA and RNA. To test the ability of these new psoralen derivatives to bind covalently to DNA, calf thymus DNA solutions were prepared according to procedures described under Materials and Methods, and then irradiated with long-wavelength UV light in the presence of different derivatives. The irradiated samples were extracted with the chloroform–isoamyl alcohol mixture and dialyzed into 0.01 M Tris–0.001 M EDTA, pH 8.4. These treatments have been shown to remove more than 95% of the radioactivity in a solution containing the new derivatives alone, with or without irradiation prior to the extraction. The extraction procedures for trioxsalen are discussed under Materials and Methods.

In order to investigate the kinetics of the photochemical reaction, DNA solutions were irradiated in the low-intensity UV irradiation (~4–6 mW/cm^2) device in the presence of one of the three psoralen derivatives, trioxsalen (**3**), 4′-hydroxy-

[2] Intensities measured with a Blak-Ray Ultraviolet Meter, Model J-221, Ultraviolet Products, Inc., San Gabriel, Calif. This meter is designed for peak response at 365 nm and has sensitivity from 300 to 400 nm.

TABLE II: Low-Intensity Photoaddition of Psoralen Derivatives in a Solution Containing 25 μg/mL DNA and a Psoralen to Base Pair Molar Ratio of 1:3.

Time of Irradiation (min)	Trioxsalen (3)		4′-Hydroxymethyltrioxsalen (5)		4′-Aminomethyltrioxsalen Hydrochloride (8)	
	a	*b*	*a*	*b*	*a*	*b*
5	19.7	15.5	89.0	3.0	15.0	19.3
10	15.5	19.7	59.6	4.6	10.5	27.4
30	13.7	22.1	26.0	10.3	6.9	41.8
60	12.8	23.6	19.5	13.8	5.9	48.7
90	10.6	28.5	17.2	15.7	5.5	52.4

[a] Moles of base pairs/moles of covalently bound psoralen. [b] Percent of added psoralen covalently bound.

TABLE III: High-Intensity Photoaddition of Psoralen Derivatives in a Solution Containing 25 μg/mL DNA and a Psoralen to Base Pair Ratio of 1:3.

Time of Irradiation (min)	Trioxsalen (3)		4′-Hydroxymethyltrioxsalen (5)		4′-Methoxymethyltrioxsalen (6)		4′-Aminomethyltrioxsalen Hydrochloride (8)	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
20	11.6	25.9	9.1	33.0	14.1	21.3	4.7	63.8
40	10.6	28.3	9.2	32.6	14.3	21.0	4.8	62.5
60	12.2	24.6	9.1	33.0	13.8	21.7	4.8	62.5

[a] Moles of base pairs/moles of covalently bound psoralen. [b] Percent of added psoralen covalently bound.

methyltrioxsalen (**5**), 4′-aminomethyltrioxsalen (**8**). The results are shown in Table II and Figure 1. It is clear that 4′-aminomethyltrioxsalen (**8**) reacts with DNA much faster than trioxsalen (**3**), which in turn has a greater initial rate of photochemical binding than 4′-hydroxymethyltrioxsalen (**5**). At an irradiation time of 90 min, the moles of drug bound per mole of base pairs are 0.18 for compound **8**, while those of **3** and **5** are 0.09 and 0.06, respectively. Table II also shows that after 90 min of irradiation, over half of the molecules of 4′-aminomethyltrioxsalen (**8**) in the solution were covalently bound to DNA, while more than 80% of 4′-hydroxymethyltrioxsalen (**5**) remains free in the solution. These differences most likely result from the influence of the molecular structures of the different psoralens on their solubilities, on their photochemical reactivities, and on the photodestruction of the compounds themselves. This will be discussed later.

How many molecules of each kind of psoralen will bind covalently to DNA if the photochemical reaction is allowed to go to completion? To answer this question, four DNA solutions each containing one of the four ^{3}H-labeled derivatives **3, 5, 6,** and **8,** were irradiated in the high-intensity UV (~100 mW/cm^2) device. Aliquots of samples were taken at time intervals of 20 min and the amount of psoralen bound covalently to DNA was determined. As can be seen from Table III, the photochemical reactions are complete after 20 min of irradiation for all of the four compounds tested. It is interesting to note that in none of the DNA–psoralen solutions have all the drug molecules become bound to the DNA, indicating either the reactions reached equilibrium or that the derivatives were photodestroyed and were no longer capable of photoreacting with the bases in the DNA. Table III lists the moles of base pairs per mole of covalently bound drugs, as well as the percent of drugs bound to the DNA. The binding decreases in the order of **8** > **5** > **3** > **6.** Thus, 4′-aminomethyl-4,5′-8-trimethylpsoralen hydrochloride (**8**) is the best derivative tested, in the sense that it can react with DNA to the extent of 0.21 mol of drug per mole of base pairs without any extra addition of free psoralen to the original DNA–drug solution during the irradiation.

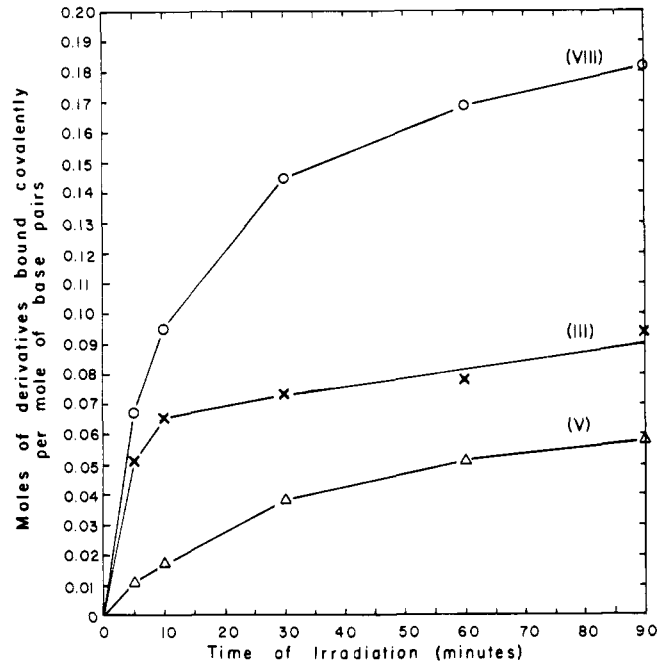

FIGURE 1: Photochemical reactions of psoralen derivatives with calf thymus DNA in low-intensity UV light device. Solutions containing calf thymus DNA and one of the psoralen derivatives were irradiated with the long-wavelength UV light (~4–6 mW/cm^2) at 10 °C for different times and the amounts of the derivatives bound to DNA were determined as described in the text. (x) 4,5′,8-Trimethylpsoralen (**3**); (Δ) 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (**5**); (O) 4′-aminomethyl-4,5′,8-trimethylpsoralen hydrochloride (**8**).

The photochemical binding of psoralen derivatives to RNA has also been studied using the four compounds (**3, 5, 6,** and **8**) and ribosomal RNA isolated from *Drosophila melanogaster* embryos. Table IV show the results of irradiating the RNA–

TABLE IV: High-Intensity Photoaddition of Psoralen Derivatives in a Solution Containing 25 μg/mL RNA and a Psoralen to Base Pair Ratio of 1:3.

Time of Irradiation (min)	Trioxsalen (3)		4′-Hydroxymethyl-trioxsalen (5)		4′-Methoxymethyl-trioxsalen (6)		4′-Aminomethyl-trioxsalen Hydrochloride (8)	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
20	23.1	13.0	26.3	11.5	264.2	1.2	5.1	59.4
40	21.6	13.9	24.8	12.1	216.8	1.4	5.3	57.2
60	20.6	14.8	23.5	13.0	183.4	1.6	5.3	57.3

[a] Moles of base pairs/moles of covalently bound psoralen. [b] Percent of added psoralen covalently bound.

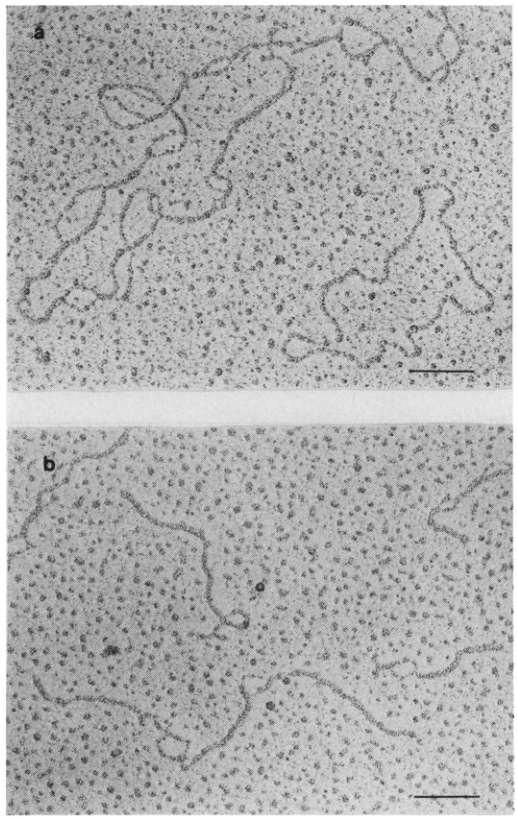

FIGURE 2: 4′-Hydroxmethyltrioxsalen (**5**) cross-linked double-stranded nucleic acids spread for electron microscopy after denaturation with glyoxal. (a) Calf thymus DNA was cross-linked with 4′-hydroxymethyltrioxsalen in the low-intensity UV device for 30 min (details described in text) and denatured with 0.5 M glyoxal. The circular molecule in the lower right part of the photograph is a single-stranded fd virus DNA with a length of about 5700 nucleotides. (b) Double-stranded reovirus-RNA solution was irradiated in the low-UV intensity device for 60 min with concentrations of 10 μg/mL RNA and 30 μg/mL 4′-hydroxymethyltrioxsalen (**5**). The cross-linked RNA was then denatured with 0.5 M glyoxal at 37 °C for 1 h and spread for electron microscopy. Magnification 50 000 times. Bars (—) in both figures represent a length of 500 base pairs.

psoralen solutions with 100 mW/cm^2 intensity UV light for 20, 40, and 60 min. The compound 4′-methoxymethyl-4,5′,8-trimethylpsoralen (**6**) bound very little, if any, to the RNA, while 4-aminomethyl-4,5′,8-trimethylpsoralen hydrochloride (**8**) reacted with RNA to an extent of about 1 mol of drug per 10 mol of nucleotides. Compounds **3** and **5** have relatively low reactivity, with approximately 1 mol of derivative bound covalently to 50 mol of nucleotides. It is important to note that the RNA samples were irradiated in 0.01 M Tris–0.001 M EDTA (pH 8.4) at 10 °C, a condition at which we believe a large amount of secondary structure exists in the RNA molecules (Wellauer and Dawid, 1973). The secondary structure may be responsible for the reaction of the psoralens with the ribosomal RNA.

Cross-linking of Nucleic Acids by the Psoralen Derivatives. Psoralen molecules have been reported to be capable of intercalating in the DNA double helix and forming interstrand bridges. This phenomenon can be detected by denaturation–renaturation kinetics (Cole, 1970, 1971), as well as by denaturation electron microscopy (Hanson et al., 1976; Wiesehahn and Hearst, 1976). We have observed, using the later method, that the psoralen derivatives **3, 5,** and **8** are all effective in cross-linking double-stranded DNA and RNA. Although the details of these studies will be presented elsewhere, some evidence is given here. Figure 2 shows electron micrographs under denaturing conditions of calf thymus DNA (Figure 2a) and double-stranded reovirus RNA (Figure 2b), both of which had been photoreacted with one of the drugs, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (**5**). The loops are the uncross-linked regions, while the apparently double-stranded regions are the places where interstrand cross-links have formed and thus prevented the two strands from separating under the denaturing conditions used for electron microscopy. Control experiments have shown that nucleic acids alone, nucleic acids + UV, or nucleic acids + derivatives appeared totally single stranded after the same denaturation treatment.

Discussion

An understanding of the photoreaction between the psoralens and DNA will provide a powerful tool in the study of nucleic acid secondary structure (Hanson et al., 1976; Wiesehahn and Hearst, 1976; Shen and Hearst, 1976). Among the most attractive features of these compounds is their ability to penetrate cells and viruses in vivo with no apparent disruption of cellular processes in the absence of electromagnetic excitation. Their chemical action on cells can be temporally and spacially controlled by the delivery of long-wavelength ultraviolet light.

A tentative mechanism for the reaction of the psoralens with DNA is as follows:

$$P + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} PS \quad (1)$$

$$PS + h\nu \xrightarrow{k_2} A \quad (2)$$

$$P + h\nu \xrightarrow{k_3} B \quad (3)$$

where P is the psoralen derivative in question, S is a psoralen binding site in the DNA or RNA, PS is the noncovalent intercalation complex between psoralen and the DNA or RNA

site, A refers to covalent adduct of the psoralen to the nucleic acid, and B is photobreakdown product of the psoralen. Despite some simplifying assumptions, a discussion of which follows, there is little doubt that these equations serve as an adequate first-order model to these reactions.

The equilibrium binding of psoralen with DNA or RNA expressed by eq 1 involves the intercalation of the planar photoreagent in the DNA or RNA helix. This equilibrium binding has been measured under conditions far enough from saturation of binding sites so that the question of site exclusion is not a factor. The concentration of binding sites [S] is proportional to the concentration of the number of base pairs and the two are taken to be equal in the equations in this paper. The dissociation constant

$$K_D = \frac{k_{-1}}{k_1} = \frac{[P][S]}{[PS]}$$

is an indication of the strength of binding of the psoralen to the DNA or RNA. This equation ignores the possible effect of base sequence upon the strength of binding.

The formation of a covalent adduct, as expressed by eq 2, requires a photon, and, since typically the quantum yield for photochemistry is wavelength dependent, the rate constant, k_2, will depend upon excitation wavelength. This equation ignores the fact that a variety of photoadducts are formed because of the potential reactivity of either thymine or cytosine with either end of the psoralen molecule. Furthermore, different geometric isomers are possible as products. When photo-cross-linkage occurs, a fraction of the photoadducts, A, absorbs a second photon and forms an additional putative cyclobutane bridge to a pyrimidine on the nucleic acid strand opposite the site of the original monoadduct. The above equations ignore this additional photochemistry, since the adduct, A, remains intact and detectable by the radioactive label in its structure.

Equation 3 refers to the photobreakdown of the free-psoralen reagent. The rate constant, k_3, will also be dependent upon the wavelength of the excitation radiation and the structure of the psoralen derivative. The breakdown products, B, may be varied in number, as well. Implicit in these three equations is the assumption that the psoralen cannot be destroyed by photoreaction while intercalated in the DNA. At present, insufficient data have been obtained to establish the validity of this point.

These equations lead to two limiting cases in the kinetics of adduct formation. In the low binding limit, the situation which pertains to trioxsalen, methoxsalen, and other commercially available psoralen derivatives, a small fraction of the potential DNA binding sites is occupied and a substantial fraction of the psoralen derivative is free in solution. By utilizing the steady-state approximation on [PS], the rate of adduct formation is given by

$$\frac{d[A]}{dt} = \frac{k_1 k_2 I[P][S]}{k_{-1} + k_2 I} \quad (4)$$

where I is the intensity of the excitation radiation. At the same conditions, the rate of formation of breakdown product is

$$\frac{d[B]}{dt} = k_3 I[P] \quad (5)$$

It follows that the ratio of rates of the two competing processes of interest is

$$\frac{\text{Rate of monoaddition}}{\text{Rate of photodestruction}} = \frac{(k_2/k_3)[S]}{K_D + (k_2/k_1)I} \quad (6)$$

Equation 6 indicates that in the low-binding limit ($K_D > k_2[S]/k_3$) high degrees of monoaddition of psoralen to DNA can only be obtained by repeated replenishment of psoralen reagent. We have observed this to be the case. In this situation, the probability of photoaddition to DNA for an intercalated psoralen and the probability of photodestruction of a free psoralen molecule in solution may be comparable in magnitude.

The light-intensity term in the denominator of eq 6 becomes important when the rate of intercalation ($k_1[P][S]$) becomes the rate-limiting step in the rate of photoaddition to the nucleic acid. In this situation, the rate of photodestruction of free psoralen remains proportional to light intensity, while the rate of photoaddition to the nucleic acid no longer is.

In the high-binding limit, the combination of low dissociation constant, K_D, and high psoralen solubility, [P], results in a situation where virtually all binding sites in the DNA are occupied prior to photochemistry, and, ideally, a very small fraction of the psoralen is free in solution. Under these conditions, the production of adduct is a simple first-order process, as expressed in eq 7, and virtually no photodestruction of photoreagent occurs.

$$[A] = [PS]_0(1 - e^{-k_2 It}) \quad (7)$$

where $[PS]_0$ is the concentration of bound sites prior to irradiation ($t = 0$).

In Table I, the properties of the newly synthesized derivatives are compared with those of trioxsalen. A summary of these data consists of the following statements.

(1) The dissociation constants for equilibrium binding to DNA show that 4′-aminomethyltrioxsalen (**8**) binds about eight times more strongly to DNA than does trioxsalen. We believe the fraction of binding which is associated with intercalation as opposed to outside binding is high, although our experiments relating to this question are not completed. The binding of trioxsalen is five times stronger than 4′-hydroxymethyltrioxsalen (**5**) and two times stronger than 4′-methoxymethyltrioxsalen (**6**). These two new derivatives still, however, bind ten times more strongly to DNA than methoxsalen (**2**), the other common commercially available psoralen.

(2) The relative solubilities of these psoralens in the order 4′-aminomethyltrioxsalen (**8**), 4′-hydroxymethyltrioxsalen (**5**), 4′-methoxymethyltrioxsalen (**6**), methoxsalen (**2**) and trioxsalen (**3**) are approximately 10 000:68:17:80:1. By dividing the molar solubility by the dissociation constant mentioned above, it can be shown that at equilibrium in a solution saturated with the respective psoralen it is not possible to saturate the DNA-binding sites with either trioxsalen or methoxsalen. The best one can do is approximately one psoralen per twenty base pairs. Column 5 in Table I shows the ratio of bound to free sites in a DNA solution saturated with the appropriate psoralen. The 4′-hydroxymethyltrioxsalen and 4′-methoxymethyltrioxsalen would be expected to nearly achieve saturation of DNA sites by binding about one psoralen per three base pairs ([PS]/[S] = 0.5), while the 4′-aminomethyltrioxsalen is predicted to be 10^4 times more effective at reaching this state of site saturation (it is 150 times more soluble than 4′-hydroxymethyltrioxsalen and binds about 100 times more strongly).

(3) Because photodestruction of the free psoralen occurs, the amount of psoralen per base pair covalently bound to DNA after delivery of a saturating amount of light depends primarily upon the fraction equilibrium bound. This number, according to eq 6, also depends upon the relative rates of photodestruction and photoreaction with the nucleic acid which appear from the

results here to be comparable. It is impossible to achieve very heavy reaction (photochemical saturation) of DNA with trioxsalen without repeated addition of fresh trioxsalen to the solution. We have observed this phenomenon with free DNA, with *Drosophila melanogaster* nuclei (Wiesehahn et al., 1977), with SV40 chromosomes (Hallick, personal communication), with *E. coli*, and with Herpes Simplex Virus I (Hanson, personal communication). The enhanced photodestruction of reagent associated with high light intensities in the low-binding limit is likely to be a more important phenomenon when reacting with organelles or intact viruses or cells, because the rate of intercalative binding can be very much slower in these cases, due to structural or steric constraints.

(4) The binding to RNA is somewhat weaker, the dissociation constants (still defined in terms of base pairs to facilitate direct comparison) are two to nine times greater than those for DNA. Because of this weaker binding, and because the photochemistry is apparently less efficient, the properties of the new derivatives, especially 4′-aminomethyltrioxsalen (**8**), make photoreaction with RNA far more effective. Column 7 of Table I shows the ratio of bound to free site in RNA for saturated psoralen solutions, clearly showing the great advantage of **8**. This greater reactivity of the aminomethyl derivative has been observed with Rous Sarcoma Virus RNA (Hallick, personal communication) and in inactivation studies on Western Equine Encephalitis Virus (Hanson, personal communication) and Vesicular Stomatitis Virus (Hearst and Thiry, in preparation).

(5) It is our intention to eventually apply the psoralen reaction to rapid kinetic studies in vivo. Such studies require intense light pulses with a duration of 10^{-3} s or less. At these times, structural changes associated with the shifting of proteins are far less likely to affect our structural analyses. In order to perfect this technique, strong binders are essential, for there is no time to wait for the new drug to intercalate in the DNA or RNA. The 4′-aminomethyltrioxsalen (**8**) and other amino alkyl derivatives which are being synthesized appear to be ideal for such study.

The results presented here are largely consistent with the model presented in this discussion. The photobinding of the various derivatives to DNA has been compared at psoralen concentrations of 2.5 μg/mL and at a base pair to psoralen ratio of 3:1. The trioxsalen is only soluble to the extent of about 0.6 μg/mL, so it is at one-fourth the soluble concentration of the other psoralens. Because it binds about five times more strongly to DNA than the 4′-hydroxymethyltrioxsalen (**5**) at these conditions, ¼ × 5 times more psoralen per base pair should be covalently bound to the DNA than with the 4′-hydroxymethylpsoralen. Figure 1 shows that this is the case. Because of the far greater solubilities of the newly synthesized compounds, conditions of higher concentration of drug would clearly enhance the advantages of these compounds over trioxsalen. Supersaturated solutions of **5** and **6** appear to be stable for many hours, making these compounds still more effective.

Only in the case of the 4′-aminomethyltrioxsalen (**8**) does one find a large fraction of the added drug covalently photoreacted with the DNA or RNA (Table III). In this case, 1 psoralen per 4.7 base pairs is photoreacted for an addition of 1 psoralen per 3 base pairs, so 65% of the added drug is covalently bound after photoexcitation. The fraction of drug equilibrium bound to the DNA under these conditions based on the K_D in Table I is about 50%. This result is thus consistent with the idea that diffusion of large amounts of additional psoralen into the DNA is not the major mechanism of the photoreaction. Such a mechanism is apparently depressed by the photodestruction of the free psoralen.

In conclusion, we have synthesized five new psoralen derivatives, three of which have solubility and binding properties to nucleic acids, which make them superior reagents for the purpose of achieving high ratios of photoadduct to base pair in the DNA. Electron microscopy has demonstrated that these reagents are effective in cross-linking DNA double helices. A similar analysis shows they are also effective in cross-linking RNA double helices, a property which has not been reported previously for the psoralens.

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