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# Psoralen-Deoxyribonucleic Acid Photoreaction. Characterization of the Monoaddition Products from 8-Methoxypsoralen and 4,5′,8-Trimethylpsoralen†

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ABSTRACT: The isolation and structural characterization are described of the major monoaddition products formed in the photoreaction of two naturally occurring psoralens, 8-methoxypsoralen and 4,5′,8-trimethylpsoralen, with high molecular weight, double-stranded DNA. Hydrolysis of the psoralen-modified DNA and subsequent chromatography resulted in the isolation of four modified nucleosides from each psoralen. Structural characterization was accomplished by mass spectrometry and $^1$H NMR analysis. The major products, accounting for 44-52% of the covalently bound psoralen, are two diastereomeric thymidine adducts formed by cycloaddition between the 5,6 double bond of the pyrimidine and the 4′,5′ (furan) double bond of the psoralen. A minor product, less than 2% of the covalently bound psoralen, is a furan-side adduct to deoxyuridine, derived from an initially formed deoxycytidine adduct by hydrolytic deamination. A fourth product is a thymidine adduct where cycloaddition has taken place between the 5,6 double bond of the pyrimidine and the 3,4 (pyrone) double bond of the psoralen. This pyrone-side adduct accounts for 19% of the covalently bound 8-methoxypsoralen but for less than 3% of the covalently bound 4,5′,8-trimethylpsoralen. All of the isolated adducts have cis-syn stereochemistry. The stereochemistry and product distribution of the adducts are determined in part by the constraints imposed by the DNA helix on the geometry of the noncovalent intercalation complex formed by psoralen and DNA prior to irradiation.

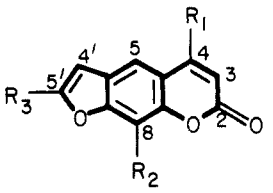
Psoralens or furocoumarins are a class of compounds found in a wide variety of plants and fungi and have been used since ancient times as dermal photosensitizing agents for the treatment of various skin pigmentation disorders (Scott et al., 1976). Their photosensitizing activity is dependent upon subsequent exposure to near-ultraviolet light (300-380 nm) and is now known to involve photochemical addition to the DNA of the target tissue (Song & Tapley, 1979). This addition to cellular DNA involves both monoaddition and interstrand cross-linking, as evidenced by denaturation-renaturation kinetics and electron microscopy (Cole, 1970, 1971; Hanson et al., 1976). The ability of psoralens to cross-link double-stranded DNA has attracted interest in their use as probes of nucleic acid structure and function (Wiesehahn et al., 1977; Shen & Hearst, 1977), and they are also under investigation as therapeutic agents for the treatment of psoriasis (Wolff et al., 1977). In addition, their ability to interact efficiently with DNA has drawn attention to their potential as naturally occurring mutagens and carcinogens. The mutagenic activity of compounds such as 8-methoxypsoralen (xanthotoxin) and 5-methoxypsoralen (bergapten) is well established, and reports have appeared documenting the tumorogenic activity of psoralens in a number of species, including man (Ashwood-Smith et al., 1980; Stern et al., 1979; Epstein, 1979).

The photoreaction between psoralens and DNA can be divided into at least three distinct steps: (1) formation of a noncovalent complex with DNA via intercalation of the psoralen between adjacent base pairs; (2) photoreaction between the psoralen and a pyrimidine base to yield a monoadduct; (3) absorption of a second photon to yield an interstrand cross-link. The interstrand cross-links are believed to be largely responsible for the photosensitizing effects of psoralen treatment, although some activity is apparently associated with the monoadducts (Harten et al., 1976). The chemistry of the photoreaction has been studied intensively, but detailed information about the structures of the nucleic acid-psoralen adducts has been notably lacking. The study reported here is concerned with the isolation and identification of the monoaddition products formed from the photobinding to DNA of

† From the Department of Chemistry and Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. *Received August 3, 1981.* This research was supported in part by the Office of Environmental Research and Development, Biomedical and Environmental Research Division, Department of Energy, under Contract No. W-7405-ENG-48, and the National Institute of General Medical Sciences (Grant GM-25151). NMR studies were carried out at the University of California, Davis, NMR Facility under the auspices of National Science Foundation Grant CHE 79-04832 or at the Stanford Magnetic Resonance Laboratory under the auspices of National Science Foundation and National Institutes of Health Grants GP-263633 and RR-00711. Mass spectrometry studies were carried out at the University of California, Berkeley, Bioorganic, Biomedical Mass Spectrometry Resource supported by National Institutes of Health Grant RR-00719, Dr. A. L. Burlingame, Director.

0006-2960/82/0421-0861$01.25/0 

two of the most widely used naturally occurring psoralens, 8-methoxypsoralen (xanthotoxin or 8-MOP, **1**) and 4,5′,8-trimethylpsoralen (trioxsalen or TMP, **2**).



**1**: $R_1 = R_3 = H$, $R_2 = OCH_3$

**2**: $R_1 = R_2 = R_3 = CH_3$

We have isolated and characterized four monoaddition products derived both from the reaction of **1** or **2** with native, double-stranded DNA. Two of the products are diastereomeric psoralen–thymidine adducts, formed by cycloaddition between the 5,6 double bond of the pyrimidine and the 4′,5′ (furan) double bond of the psoralen. A third and minor product (less than 2% of the isolated monoadducts) is a furan-side psoralen–deoxyuridine adduct, derived from an initially formed psoralen–deoxycytidine adduct by hydrolytic deamination. A fourth monoadduct recovered from both 8-MOP- and TMP-modified DNA has been characterized as an adduct resulting from cycloaddition of thymidine residues to the 3,4 (pyrone) double bond of the psoralen. In the case of 8-MOP, this pyrone-side adduct accounts for 19% of the total covalently bound psoralen, while in the case of TMP only 2–3% of the bound psoralen is present as pyrone-side adducts.

## Experimental Section

*Materials.* 8-[$^3$H]Methoxypsoralen and 4,5′,8-[$^3$H]trimethylpsoralen were synthesized as described (Isaacs et al., 1982). Hydrolytic enzymes and calf thymus DNA were obtained from Sigma Chemical Co. (St. Louis, MO).

*Photobinding.* A 4.05-mg sample of [$^3$H]-8-MOP (sp act. 0.1–0.5 Ci mmol$^{-1}$) in 1.5 mL of ethanol was added to a solution of 43.2 mg of calf thymus DNA in 60 mL of Tris buffer (10 mM Tris, pH 7.2). This solution was irradiated for 2.5 h at 20 °C. Photobinding of [$^3$H]TMP (0.1–0.5 Ci mmol$^{-1}$) to DNA was carried out by adding five 300-μL aliquots of a stock solution (1.25 mg of TMP in 1.5 mL of ethanol) to the DNA solution (7.2 mg of DNA at a concentration of 0.12 mg mL$^{-1}$ in Tris buffer). The solution was irradiated for 10 min after each of the psoralen additions. Two 400-W GE mercury vapor lamps were used for the irradiation, and cooling of the DNA solution was achieved by circulation of a solution of cobaltous nitrate (40% w/w) through an outer jacket; this solution also served as a 365-nm transmission filter. The intensity of light at the surface of the inner sample chamber in this device is approximately 100 mW/cm$^2$ (Isaacs et al., 1977). After the appropriate irradiation time, the psoralen–DNA solution was extracted with 4 volumes of chloroform to remove unreacted psoralen and its photodegradation products. The remaining solution was made 0.2 M in sodium chloride and diluted with 3 volumes of cold ethanol to precipitate the DNA which was collected by centrifugation, dissolved in 0.2 M sodium chloride, and reprecipitated by addition of ethanol. The isolated DNA pellet was then dried under vacuum and redissolved in hydrolysis buffer (15 mM sodium acetate, pH 5.00). Alternatively, the DNA was subjected to acid hydrolysis, as described below.

*Adduct Isolation.* The psoralen-modified DNA was first hydrolyzed by the addition of 80 units of DNase II (EC 3.1.22.1) per mg of DNA. After 12 h, the solution was adjusted to pH 7, and 0.2 unit of phosphodiesterase II (EC 3.1.4.18) per mg of DNA was added. After an additional 12 h, the pH was adjusted to 8.0, and 0.2 unit of alkaline phosphatase (EC 3.1.3.1) per mg of DNA was added. After a total hydrolysis time of 24–36 h, the mixture was lyophilized and redissolved in water. This mixture was then analyzed by HPLC. HPLC was carried out on reverse-phase Ultrasphere ODS columns (either 4.6 or 10 mm × 25 cm; Altex-Beckmann, Berkeley, CA). Water–methanol was used as the eluting solvent, at a flow rate of 1 or 4 mL min$^{-1}$. In cases where weak ion-exchange effects led to peak broadening, a buffered aqueous phase was used (10 mM $KH_2PO_4$, pH 2). The column effluent was monitored for absorbance at 254 nm and assayed for the presence of $^3$H by scintillation counting.

*Mass Spectrometry.* Adduct fractions isolated by HPLC were taken to dryness and either analyzed directly by field desorption mass spectrometry or derivatized and then analyzed by high-resolution electron-impact mass spectrometry. Samples were converted to pertrimethylsilyl ethers by heating 0.5–2.0 μg of adduct in 50 μL of pyridine–BSTFA [*N,O*-bis(trimethylsilyl)trifluoroacetamide, 1:4 v/v] at 60 °C for 40 min under nitrogen.

Mass spectra were recorded on a modified Kratos/AEI MS902, interfaced to a Sigma 7-LOGOS II data system (Burlingame et al., 1974). Field desorption spectra were obtained by using conventional benzonitrile-activated emitters (Beckey, 1977). Samples analyzed by electron impact mass spectrometry were admitted to the ion source via the direct insertion probe (source temperature 250 °C). Electron-impact mass spectra were recorded at a dynamic resolution of 10000 (scan rate 8 or 16 s/decade), and elemental compositions were then generated for a given error tolerance for all fragment ions in the mass spectrum.

*$^1$H NMR.* $^1$H NMR spectra were recorded on a Nicolet Technologies NT-360 spectrometer or on a Bruker HXS-360 spectrometer. Typically, 256–2048 transients were accumulated at a spectral width of 1500 Hz (16K data points). For nuclear Overhauser effect experiments, the decoupling field was gated off during data acquisition, and a delay time of 3–3.5 s was inserted between the end of data acquisition and the beginning of the following pulse. With samples where the size of the residual solvent signal (HDO) was a problem, a weak saturating pulse was applied prior to the main rf pulse in order to selectively suppress the solvent signal. Spectra used for detecting NOE enhancements were first zero-filled to give 32K data points and then subtracted from the reference spectrum, usually a spectrum with the decoupling field set symmetrically on the opposite side of the carrier frequency. All assignments were made with the aid of extensive homonuclear decoupling experiments.

Spectra were recorded in either 99.996% $D_2O$ or 99.96% $Me_2SO$-$d_6$. Samples were prepared by dissolution in 99.96% $D_2O$ and then evaporation to dryness under reduced pressure. This was repeated a total of 4 times. All final loading steps were carried out under a dry nitrogen atmosphere. Typical sample concentrations ranged from 50–500 μM. Chemical shifts are referenced to $Me_4Si$ ($\delta_{HDO} = 4.75$).

*Acid Hydrolysis.* DNA samples were dissolved in 0.4 N HCl (1 mg mL$^{-1}$) and heated to 75 °C for 4 h. The hydrolyzed mixture was reduced to a minimal volume and applied to a C-18 Sep Pak cartridge (Waters Associates, Milford, MA). Elution was carried out with water and then with methanol, and the methanol fraction was analyzed by HPLC.

## Results

*HPLC and UV.* The results of the photobinding of 8-MOP and TMP to double-stranded, high molecular weight DNA

Table I: $^3$H Recoveries and Modification Levels for Psoralen-DNA Reactions

| | 8-MOP[a] | TMP[b] |
|---|---|---|
| psoralen added per base pair | 0.28 | 0.50 |
| psoralen bound per base pair | 0.065 | 0.110 |
| HPLC fractions (%[c] bound $^3$H), enzyme-hydrolyzed psoralen-DNA | F32[d] (26) | F34–36[d] (39) |
| | F36[e] (2) | F47[e] (<2) |
| | F37[f] (16) | F49[f] (52) |
| | F38[f] (28) | |
| | F41[g] (19) | F52[g] (2.5) |

[a] Single addition of psoralen; 2.5-h irradiation. [b] Multiple additions of psoralen; 10-min irradiation after each addition. [c] Average of three determinations. [d] Nucleoside-psoralen-nucleoside cross-linked fraction. [e] Furan-side deoxyuridine monoadducts. [f] Diastereomeric furan-side thymidine monoadducts. [g] Pyrone-side thymidine monoadducts.

are shown in Table I. The modification levels of 65 and 110 psoralen residues per 1000 base pairs, respectively, are similar to the levels reported in the literature by others (Wiesehahn et al., 1977). The psoralen-modified DNAs were then hydrolyzed enzymatically and the resulting hydrolysis mixture contained the psoralen-modified nucleosides, along with a much larger amount of unmodified nucleosides. Isolation of the individual adducts from this complex sample matrix was accomplished by high-performance liquid chromatography (HPLC).

Parts a and b of Figure 1 show the HPLC elution profiles obtained from enzymatically hydrolyzed [$^3$H]-8-MOP–DNA and [$^3$H]TMP–DNA, respectively. The peaks eluting between 5 and 20 min do not contain $^3$H and are related to the unmodified nucleosides (thymidine, deoxycytidine, deoxyguanosine, and deoxyadenosine). The major $^3$H-containing peaks in the case of 8-MOP-modified DNA are F32, F36, F37, F38, and F41 (Figure 1a and Table I). Unreacted 8-MOP elutes at 51 min in this chromatographic system. These five fractions together account for greater than 90% of the covalently bound $^3$H. Similarly, TMP-modified DNA yielded four major $^3$H-containing fractions; these are referred to as F35, F47, F49, and F52. Unreacted TMP has a retention time of 62 min under these conditions (Figure 1b and Table I).

The fractions with elution times of between 36 and 55 min in this chromatographic system contain psoralen–nucleoside monoadducts, and it is the characterization of these monoadducts that is the subject of this report. With both 8-MOP and TMP, substantial amounts of interstrand cross-links are formed during these irradiations. These nucleoside–psoralen–nucleoside adducts have elution times of between 28 and 35 min, and the structures of these diadducts are currently under investigation.

The absorption spectra of the isolated fractions from 8-MOP- or TMP-modified DNA were found to be of two distinct types. Adducts F36, F37, and F38 from 8-MOP and TMP adducts F47 and F49 had very similar absorption spectra with a characteristic maxima at 328 nm. This spectrum is that of 4′,5′-dihydropsoralen (coumarin) chromophore and indicates that reaction has occurred at the 4′,5′ double bond (Musajo & Rodighiero, 1972). The remaining monoadducts (8-MOP-F41 and TMP-F52) had absorption spectra with the 328-nm band absent. This spectrum is that of a 3,4-dihydropsoralen (benzofuran) derivative and indicates that reaction has taken place at the 3,4 (pyrone) double bond of the psoralen.

*Mass Spectrometry.* The four 8-MOP–nucleoside HPLC fractions (F36, F37, F38, and F41) and the three TMP–nucleoside fractions (F47, F49, and F52) were analyzed by mass

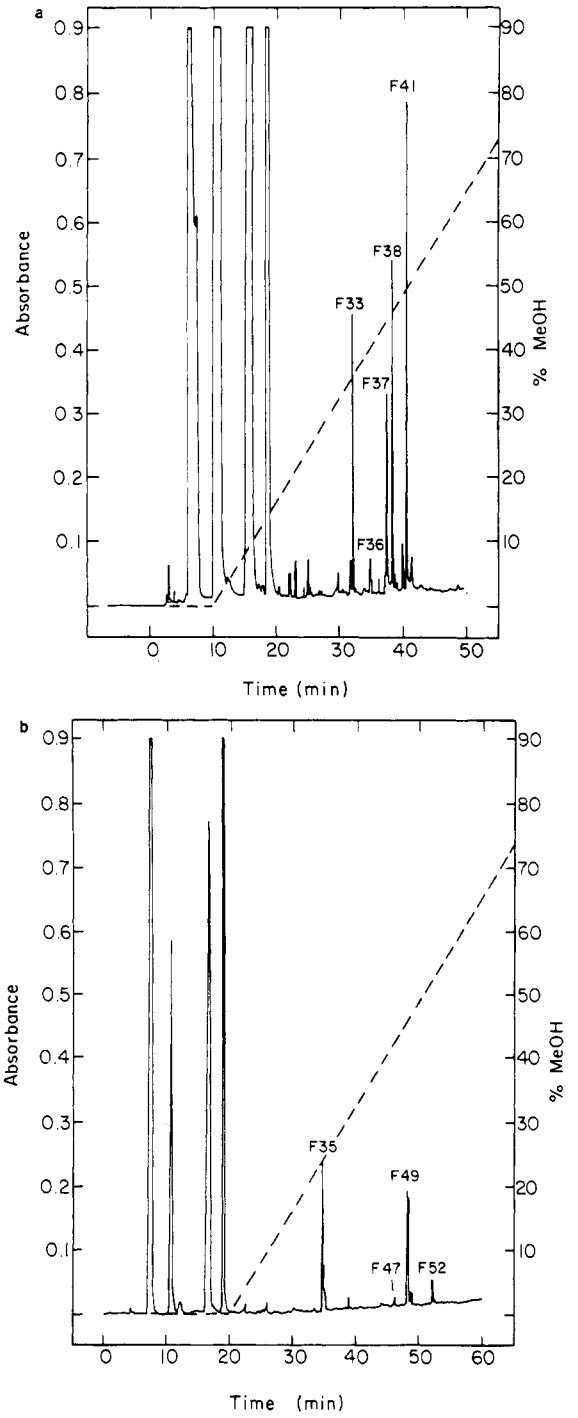



FIGURE 1: HPLC elution profile of (a) enzyme hydrolyzed 8-MOP-modified DNA and (b) enzyme hydrolyzed TMP-modified DNA.

spectrometry. The high polarity and thermal lability of nucleosides generally rule out the direct use of conventional electron-impact techniques. Molecular weight information was therefore obtained by the use of field desorption mass spectrometry (FDMS). The adducts were then converted to *N*,-*O*-pertrimethylsilyl ethers and analyzed by high-resolution electron-impact mass spectrometry. The resulting fragmentation pattern and elemental composition information, together with the molecular weight information from FDMS, allowed the assignment of overall structures to the adducts.

Analysis by FDMS gave identical results for 8-MOP fractions F38 and F37, indicating a molecular weight of 458 (Table II). 8-MOP-F36 gave results consistent with a molecular weight of 444. Analysis of the TMP adducts indicated a molecular weight of 470 for TMP-F49 and 456 for TMP-

Table II: High-Resolution Mass Spectral Data for 8-MOP-F38[a] ($Me_3Si$ Ether)

| m/z | composition | assignment |
|---|---|---|
| 587.1843 | $C_{21}H_{17}O_9N_2(Me_3Si)_2$ | $(M-CH_3)^+$ ion = m |
| 557.1749 | $C_{20}H_{15}O_8N_2(Me_3Si)_2$ | m − $CH_2O$ |
| 543.1617 | $C_{19}H_{13}O_8N_2(Me_3Si)_2$ | |
| 515.1657 | $C_{18}H_{13}O_7N_2(Me_3Si)_2$ | |
| 497.1381 | $C_{21}H_{16}O_8N_2(Me_3Si)_2$ | m − HO-$Me_3Si$ |
| 469.1436 | $C_{20}H_{16}O_7N_2Me_3Si$ | |
| 441.1484 | $C_{19}H_{16}O_6N_2Me_3Si$ | M − $C_3H_4O_3Me_3Si$ |
| 415.1327 | $C_{17}H_{14}O_6N_2Me_3Si$ | |
| 343.0904 | $C_{17}H_{15}O_6N_2$ | M − dR |
| 298.0894 | $C_{12}H_8O_4Me_3Si$ | 8-MOP + $Me_3Si$ |
| 216.0431 | $C_{12}H_8O_4$ | 8-MOP |
| 201.0185 | $C_{11}H_5O_4$ | 216 − $CH_3$ |
| 188.0473 | $C_{11}H_8O_3$ | 216 − CO |
| 173.0242 | $C_{10}H_5O_3$ | |

[a] FDMS of 8-MOP gave m/z 216, 458 ($M^{+\cdot}$ + 1), 481 ($M^+Na^+$), and 939 ($M^+Na^+$). [b] Errors for composition matches are <10 ppm.

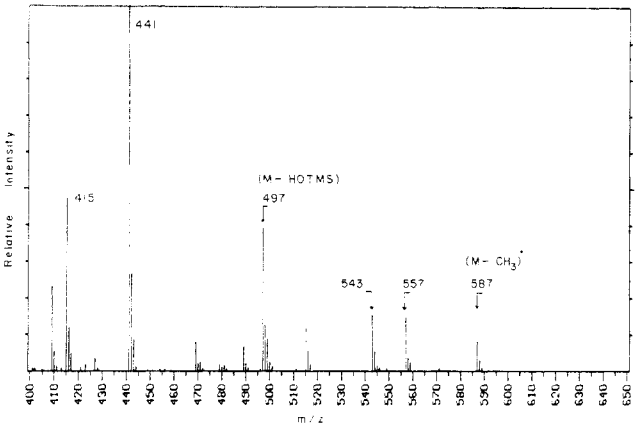



FIGURE 2: High-resolution mass spectrum (m/z 400–650) of the $Me_3Si$ ether derivative of 8-MOP-F38.

F47. There was insufficient material present in the 8-MOP-F41 or TMP-F52 fractions to obtain an FD mass spectrum.

A region of the high-resolution electron impact mass spectrum of the $Me_3Si$ derivative of 8-MOP-F38 is shown in Figure 2; 8-MOP-F37 gave an identical high-resolution spectrum, indicating that the two compounds are probably diastereomers. The highest observed mass occurs at m/z 587 [$C_{21}H_{17}O_9N_2(Me_3Si)_2$] and corresponds to the loss of a methyl radical from the molecular ion. Additional fragmentation reactions include loss of the elements of $CH_2O$ (m/z 557) and $C_2H_4O$ (m/z 543) from m/z 587. Fragmentations which are characteristic of $Me_3Si$ derivatives of nucleosides include the loss of HO–$Me_3Si$ (m/z 497) and cleavage of the C–N glycosidic bond with hydrogen rearrangement to yield the psoralen-base fragment at m/z 343 ($C_{17}H_{15}O_6N_2$). The region below m/z 220 is dominated by a set of fragment ions identical with those observed in the mass spectrum of 8-MOP. The base peak in the spectrum occurs at m/z 216. The nitrogen content ($N_2$) and overall fragmentation pattern of these two adducts are consistent with a 1:1 8-MOP–deoxythymidine adduct. The 3′- and 5′-hydroxyls of the deoxyribose have been converted to $Me_3Si$ ethers, while the pyrimidine and lactone moieties remain intact under the derivatization conditions used. The molecular weight obtained by FDMS (458) is consistent with this assignment.

While the mass spectral results alone do not directly identify the site of reaction in the psoralen, together with the UV absorption spectra of the adducts (indicating a 4′,5′-dihydropsoralen-type chromophore), the data strongly suggest a 1:1 8-MOP–thymidine adduct formed via cycloaddition to the 4′,5′ double bond of the psoralen.

The HRMS results obtained on 8-MOP-F36 were very similar to those obtained on F37 and F38, except that all of the masses above m/z 300 are shifted to lower mass by 14 units. The elemental compositions of the various fragment ions indicates that the nucleoside base moiety contains $C_4H_3N_2O_2$, ruling out the presence of either thymine or cytosine. The molecular weight [FDMS gave m/z 216, 444 ($M^{+\cdot}$), and 467 ($M^+Na^+$)] and elemental composition data are consistent with the presence of uracil. Therefore 8-MOP-F36 is a 1:1 8-MOP–deoxyuridine adduct, apparently formed by hydrolytic deamination of an initially formed deoxycytidine–psoralen adduct. This reaction has been observed in other 5,6-dihydrocytosine-type compounds (Freeman et al., 1965). The absorption spectrum of F36 indicates that cycloaddition has occurred at the 4′,5′ double bond of the psoralen.

The results obtained on the major TMP–DNA adducts (F49 and F47) indicate that F49 is a 1:1 thymidine–TMP adduct and that F47 is a 1:1 deoxyuridine–TMP adduct. In both cases the mass spectrum is dominated by fragment ions from the TMP moiety (base peak = m/z 228), and the region below m/z 300 is very similar to the spectrum of TMP itself. The highest observed mass for F49 occurs at m/z 599 [$C_{23}H_{21}O_8N_2(Me_3Si)_2$], corresponding to loss of a methyl radical from the molecular ion. The fragmentation pattern is similar to that observed with the 8-MOP adducts and includes ions derived from loss of the elements of $CH_2O$ (m/z 569), $C_2H_4O$ (m/z 555), and HO–$Me_3Si$ (m/z 509) and cleavage of the glycosidic bond (m/z 355). The FDMS results on F49 indicated a molecular weight of 470. The $Me_3Si$ derivative of TMP-F47 gave a similar set of fragments, with the masses above m/z 310 shifted to lower mass by 14 units. This result, along with the molecular weight information obtained by FDMS [m/z 228, 456 ($M^{+\cdot}$), 479 ($M^+Na^+$), and 935 ($M_2^+Na^+$)], is consistent with its assignment as a 1:1 deoxyuridine–TMP adduct. The absorption spectra of these adducts are consistent with cycloaddition having occurred at the 4′,5′ double bond of the psoralen.

The remaining products, 8-MOP-F41 and TMP-F52, gave high-resolution mass spectra (as $Me_3Si$ ethers) markedly different from that of the other adducts. For TMP-F52 the base peak in the spectrum occurred at m/z 390 [$C_{14}H_{12}O_4(Me_3Si)_2$] rather than m/z 228. This suggests that hydrolysis and derivatization of the lactone moiety has occurred, resulting in a $(Me_3Si)_2$ derivative of TMP. An additional fragment at m/z 386 [$C_{10}H_{12}O_5N_2(Me_3Si)_2$] indicates the presence of thymidine. These results are consistent with a 1:1 TMP–thymidine adduct in which the lactone moiety of the psoralen has undergone hydrolysis to the hydroxy acid and subsequent conversion to a $Me_3Si$ ether/$Me_3Si$ ester. Facile ring opening of the lactone is characteristic of a 3,4-dihydropsoralen, and the absorption spectrum of this adduct is consistent with the absence of a 3,4 double bond. TMP-F52 is therefore most probably a 1:1 thymidine–TMP adduct, where cycloaddition has occurred on the pyrone (3,4 double bond) side of the psoralen. Extensive fragmentation apparently takes place with the $(Me_3Si)_4$ derivative of this adduct, so that few ions directly related to the molecular ion are observed. The highest observed mass occurs at m/z 516 [$C_{19}H_{18}O_6N_2(Me_3Si)_2$] and corresponds to cleavage of the glycosidic bond. There was insufficient material available for FDMS or $^1H$ NMR; further work to confirm the identity of this adduct is in progress.

The mass spectrum of the $Me_3Si$ derivative of 8-MOP-F41 was similar to that of TMP-F52 in that ions diagnostic for the

presence of a $(Me_3Si)_2$ derivative of the psoralen occur at $m/z$ 378 $[C_{12}H_8O_5(Me_3Si)_2]$ and 363 $[C_{11}H_5O_5(Me_3Si)_2]$. Additional ions indicating the presence of thymidine occur at $m/z$ 386 $[C_{10}H_{12}N_2O_5(Me_3Si)_2]$ and 126 $(C_5H_6N_2O_2)$. These data, together with the ultraviolet absorption spectrum, are consistent with the presence of a 1:1 8-MOP–thymidine adduct where cycloaddition has occurred at the 3,4 (pyrone) double bond of the psoralen.[1]

The psoralen–DNA cycloaddition which occurs at the 5,6 double bond of a pyrimidine base greatly facilitates the susceptibility toward acid hydrolysis of the C–N glycosidic bond of the modified nucleoside (Cohn & Doherty, 1956). Acid hydrolysis of the psoralen-modified DNA was therefore used to isolate and characterize the aglycon derivatives of the psoralen-modified residues.[2] Hydrolysis of 8-MOP–DNA in mild acid (0.4 N HCl; 75 °C; 4 h) and subsequent chromatography yielded three monoadducts, referred to as 8-MOP-F39H$^+$, 8-MOP-F40H$^+$, and 8-MOP-F42H$^+$, in approximately a 5:80:15 ratio. The absorption spectrum of the major product, 8-MOP-F40H$^+$, indicated the presence of a 4′,5′-dihydropsoralen chromophore and is different from that of the other two fractions. 8-MOP-F39H$^+$ and 8-MOP-F42H$^+$ were found to interconvert at room temperature and neutral pH; at elevated temperatures in mild acid (50 °C; pH 2), the formation of F42H$^+$ is favored over F39H$^+$. F42H$^+$ has an absorption spectrum similar to that of TMP-F52, indicating that reaction has occurred at the 3,4 (pyrone) double bond of the psoralen. This interconverion between F39H$^+$ and F42H$^+$ is most likely due to hydrolysis of the lactone to a $\delta$-hydroxy acid. Both MS and NMR results obtained on F42H$^+$ are consistent with the presence of a lactone moiety.

These aglycon adducts were sufficiently volatile to allow direct HRMS analysis without the need for conversion to $Me_3Si$ derivatives. In the case of 8-MOP-F40H$^+$, a small molecular ion (<5% of base peak) was observed at $m/z$ 342 $(C_{17}H_{14}N_2O_6)$. A molecular ion was not observed for 8-MOP-F42H$^+$. Both spectra are otherwise very similar to that observed for free 8-MOP, with the exception of ions at $m/z$ 126 $(C_5H_6N_2O_2)$ and 83 $(C_4H_5NO)$. These two ions are diagnostic for the presence of thymine (McCloskey, 1974). The fragmentation patterns are therefore consistent with furan-side or pyrone-side 1:1 8-MOP–thymine adducts. The FD mass spectrum of 8-MOP-F40H$^+$ exhibited an intense signal at $m/z$ 342 ($M^+\cdot$), along with smaller signals at $m/z$ 126, 216, and 365 ($M^+Na^+$). The FD mass spectrum of 8-MOP-F42H$^+$ gave $m/z$ 216 and 126.

Acid hydrolysis of TMP-modified DNA resulted in a single major monoadduct, TMP-F50H$^+$. The mass spectrum of this adduct gave a molecular ion at $m/z$ 354 $(C_{19}H_{18}N_2O_5)$, along with ions indicative of the presence of thymine ($m/z$ 126, $C_5H_6N_2O_2$; $m/z$ 83, $C_4H_5NO$) and TMP ($m/z$ 228, 200, and 185). The FD mass spectrum of this adduct confirmed the molecular weight as 354; additional signals were observed at $m/z$ 228, 126, and 377 ($M^+Na^+$). These results are consistent with a 1:1 TMP–thymine adduct. The UV absorption spectrum was identical with that of a 4′,5′-dihydro-TMP derivative.

The relationship of the major acid hydrolysis products to the nucleoside–psoralen adducts obtained by enzyme hydrolysis

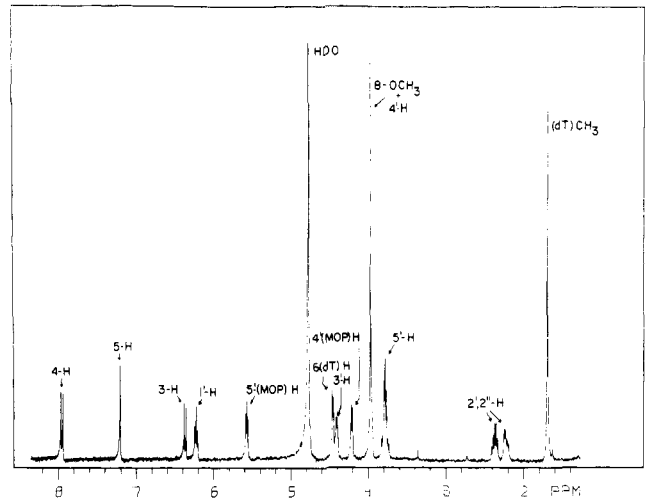



FIGURE 3: 360-MHz $^1$H NMR spectrum of 8-MOP-F37.

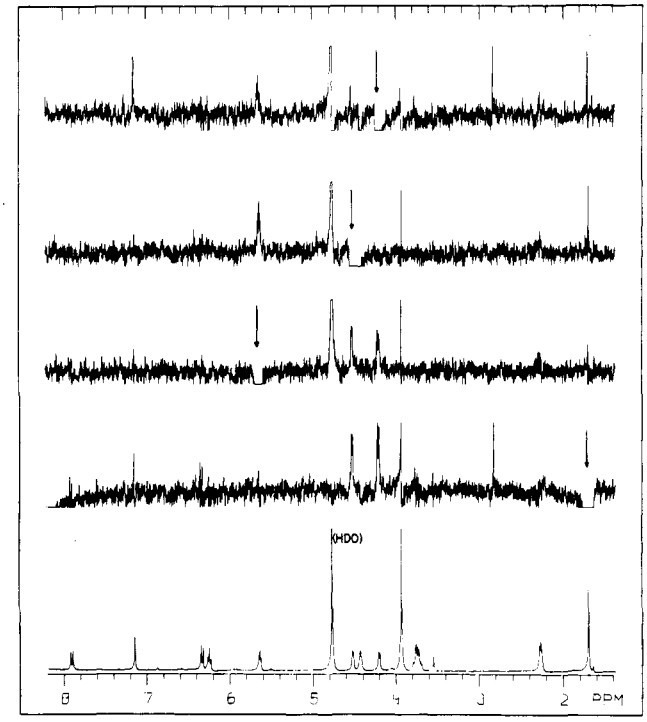



FIGURE 4: 360-MHz $^1$H-NMR spectrum of 8-MOP-F38 and NOE difference spectra for this adduct. Arrows refer to the location of the decoupling field.

was determined by subjecting the pure nucleoside adducts to identical hydrolysis conditions (0.4 N HCl; 75 °C; 4 h). Subsequent chromatography revealed that 8-MOP-F40H$^+$ is derived from both 8-MOP-F37 and 8-MOP-F38, while 8-MOP-42H$^+$ is derived from 8-MOP-F41. The major TMP–DNA acid hydrolysis product, TMP-F40H$^+$, is derived from TMP-F49. Various minor products were observed in the monoadduct fraction of the acid hydrolysates of 8-MOP- or TMP-modified DNA but were not present in amounts that allowed characterization. It is possible that the aglycon derivatives of 8-MOP-F36, TMP-F47, and TMP-F52 were present in these uncharacterized fractions.

*$^1$H NMR. (1) 8-MOP–DNA Adducts.* The 360-MHz $^1$H NMR spectra of F37 and F38 are shown in Figures 3 and 4 (see also Table III). Both adducts give similar spectra, with various small shift differences. The 4′ and 5′ protons of the 8-MOP moiety and the 5-$CH_3$ and 6-H of the thymidine residue show upfield shifts, indicating that the 4′,5′ (furan) double bond of the psoralen and the 5,6 double bond of the

[1] This thymidine adduct derives primarily from photoaddition to a thymidine residue. However, should any adduct formation have occurred with the small amount of 5-methylcytosine present (Ehrlich & Wang, 1981), it would have been hydrolytically deaminated (Freeman et al., 1965) and appear as a thymidine adduct.

[2] Those adducts obtained by acid hydrolysis are indicated by the addition of H$^+$ to the fraction number.

Table III: Chemical Shifts ($\delta$)[a] and Coupling Constants of 8-MOP-DNA Monoadducts

| moiety[b] | F36 | F37 | F38 | F41 | F40H$^+$ | F42H$^+$ |
|---|---|---|---|---|---|---|
| C-4 H (8-MOP) | 8.044 d | 7.930 d | 7.865 d | 4.161 d | 7.899 d | 3.59 d |
| $J_{4,3}$ (Hz) | 9.5 | 9.5 | 9.5 | 9.7 | 9.5 | 10 |
| C-5 H (8-MOP) | 7.181 s | 7.182 s | 7.103 s | 7.284 s | 7.131 s | 7.220 s |
| C-3 H (8-MOP) | 6.339 d | 6.343 d | 6.294 d | 3.54 dd | 6.317 d | 3.499 dd |
| C-1′ H (dR) | 6.208 t | 6.200 t | 6.210 t | 6.280 dd | | |
| C-5′ H (8-MOP) | 5.569 t | 5.546 dd | 5.600 t | 7.603 d | 5.581 t | 7.720 d |
| $J_{5',4'}$ (Hz) | 5.4 | 5.5 | 5.5 | 2.2 | 5.5 | 2.2[c] |
| $J_{5',6}$ (Hz) | 9.2 | 5.8 | 5.5 | | 5.5 | |
| C-6 H (U or T) | 4.519 m | 4.435 dd | 4.480 dd | 4.255 d | 4.155 dd | 4.015 d |
| $J_{4',6}$ (Hz) | 1.5 | 1.2 | 1.5 | | 2.5 | 10[d] |
| $J_{5,6}$ (Hz) | 9.2 | | | | | 2[e] |
| C-3′ H (dR) | 4.215 m | 4.385 m | 4.390 m | 4.358 m | | |
| C-4′ H (8-MOP) | 4.773 m | 4.189 d | 4.161 d | 6.753 d | 4.190 d | 6.640 |
| $J_{4',5}$ (Hz) | 5.4 | | | | | |
| C-5 H (U) | 4.011 t | | | | | |
| C-4′ H (dR) | 3.853 m | 3.931 m | 3.900 m | 3.901 m | | |
| C-8 $OCH_3$ | 3.894 s | 3.946 s | 3.880 s | 4.032 s | 3.963 s | 3.899 s |
| C-5′ H (dR) | 3.710 m | 3.757 m | 3.700 m | 3.714 m | | |
| C-2′,2″ H (dR) | 2.103, 1.958 m | 2.450, 2.300 m | 2.232 m | 2.08 m | | |
| C-5 $CH_3$ (T) | | 1.669 s | 1.655 s | 1.605 s | 1.655 s | 1.540 s |

[a] $\delta_{HDO}$ 4.75. [b] dR = deoxyribose; U = uracil; T = thymine. [c] $J_{4',5'}$ value. [d] $J_{3,6}$ value. [e] $J_{4,6}$ value.

Table IV: Nuclear Overhauser Effect (NOE) Experiments on Psoralen-DNA Monoadducts

| psoralen | irradiated resonance | observed resonance | % enhancements[a] F37[b] | F38[b] | F40H$^+$[c] |
|---|---|---|---|---|---|
| 8-MOP | C-5′ H (8-MOP) | C-4′ H (8-MOP) | 8.7 | 4.5 | 8.2 |
| | | C-6 H (T) | 10 | 4.7 | 4.6 |
| | | C-5 $CH_3$ (8-MOP) | 0 | 0 | 0 |
| | C-5 $CH_3$ (T) | C-4′ H (8-MOP) | 13 | 15 | 11.2 |
| | | C-6 H (T) | 9.4 | 10.6 | 10.4 |
| | | C-5′ H (8-MOP) | 0 | 0 | 0 |
| | | | | F50H$^+$[c] | |
| TMP | C-5′ $CH_3$ (TMP) | C-4′ H (TMP | | 16.7 | |
| | | C-6 H (T) | | 15.6 | |
| | C-5 $CH_3$ (T) | C-4′ H (TMP) | | 17.8 | |
| | | C-6 H (T) | | 14.8 | |

[a] Error is ±1% for F38, F40H$^+$, and F50H$^+$ and ±2% for F37. [b] Determined in $D_2O$. [c] Determined in $Me_2SO$-$d_6$.

pyrimidine have undergone reaction. These data are consistent with a cyclobutane structure derived from a 2 + 2 cycloaddition of a thymidine residue to the 4′,5′ double bond of 8-MOP.

The regiospecificity of these furan-side monoadducts can be assigned by homonuclear decoupling analysis of the protons of the cyclobutyl system. The downfield 5′-H of the psoralen is coupled equally to both the 4′-H of the psoralen and the 6-H thymidine proton, resulting in an apparent triplet at 5.55 ($J_{4',5'} = J_{5',6}$ = 5.5 Hz). This pattern is consistent only with a syn orientation about the cyclobutane ring; in the anti orientation the 5′-H psoralen proton would undergo vicinal coupling only to the psoralen 4′-H. There is a small cross coupling ($^4J$) between the protons positioned diagonally to each other on the cyclobutane ring (4′-H of the psoralen and 6-H of the thymidine). This cross coupling is readily observed for the 6-H thymidine resonance (a doublet of doublets), but in the case of the psoralen 4′-H resonance, it is masked by $^4J$ coupling between 4′-H and 5-H of the psoralen. Irradiation of the 5-H resonance yields a decoupled spectrum in which the mutual 1.5-Hz cross coupling is clearly revealed.

The cyclobutane ring can adopt a wide range of conformations, depending on the substituents present. While $^3J_{cis}$ is generally larger than $^3J_{trans}$, the ranges that these coupling constants can assume have considerable overlap ($^3J_{cis}$ = 4.6–11.4 Hz; $^3J_{trans}$ = 2.24–10.7 Hz) (Fleming & Williams, 1967). The assignment of cis or trans stereochemistry cannot therefore be made on the basis of evidence derived solely from coupling constants. Nuclear Overhauser effect (NOE) experiments, however, are ideally suited for distinguishing such stereochemistries. For a proton–proton spin system, the principal mode of relaxation is through a dipole–dipole interaction that has an $r^{-6}$ dependence on the spatial separation of the observed and irradiated spins (Bell & Saunders, 1969). The cyclobutyl systems considered in this study are ideally suited for NOE enhancement experiments since the molecule is fairly rigid in the region of interest, minimizing complications due to rotation about carbon–carbon bonds. In addition, proton–proton and proton–methyl distances in diastereomeric cis or trans fused ring systems will be substantially different, so that the $r^{-6}$ dependence of the NOE will lead to easily distinguishable differences. NOE enhancements are thus a sensitive indicator of relative stereochemistry in a substituted cyclobutane.

The NOE difference spectra for 8-MOP-F38 are shown in Figure 4. Irradiation of the 5′-H resonance in F38 resulted in approximately equal enhancements of the 6-H (dT) and 4′-H resonances (Table IV). This result not only corroborates the assignment of syn stereochemistry as derived above from a consideration of the coupling pattern but also establishes that these three protons [5′-H, 4′-H, and 6-H (dT)] are cis with respect to the plane of the cyclobutane ring. The 5-$CH_3$ of the thymidine moiety was shown to be adjacent and cis to 4′-H and 6-H (dT) when irradiation of this methyl led to a sub-

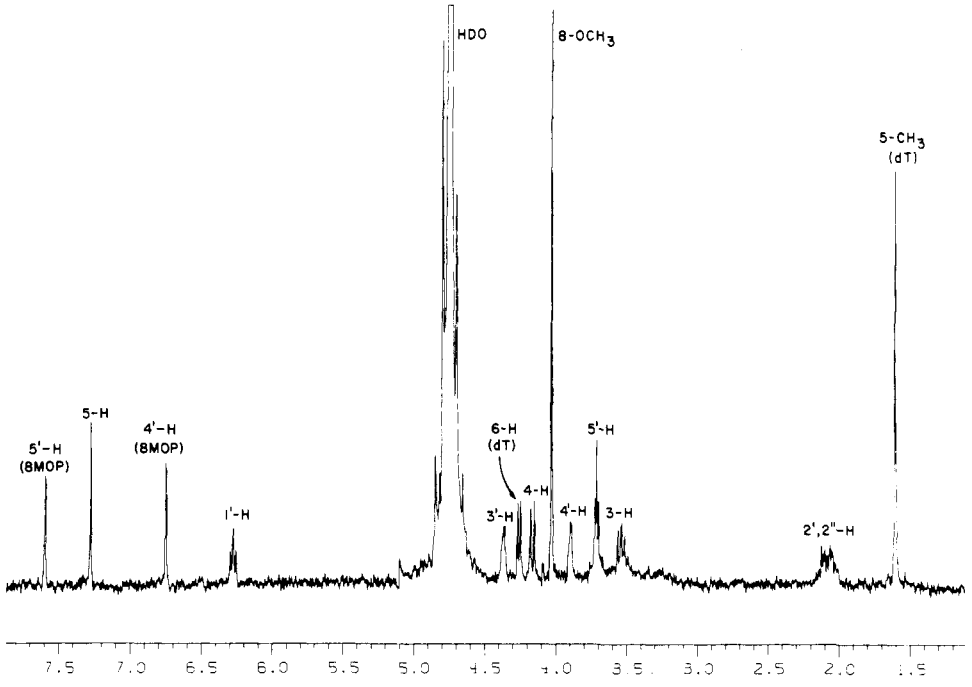
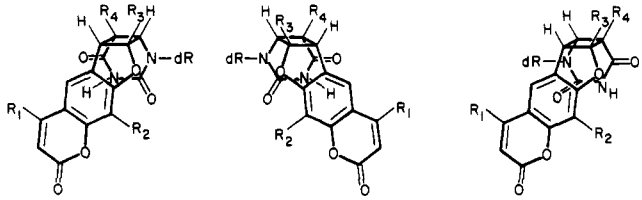



FIGURE 5: 360-MHz $^1$H NMR spectrum of 8-MOP-F41.

stantial enhancement of these two resonances. No significant NOE was observed between the 5-$CH_3$ of the thymidine moiety and 5'-H of the 8-MOP residue. These results are consistent only with a cis–syn stereochemistry, as shown in structures **3** and **3'**. Virtually identical NOE results were obtained with 8-MOP-F37, indicating that these two adducts must have the stereochemical relationship shown in structures **3** and **3'**. Additional support for the existence of cis–syn

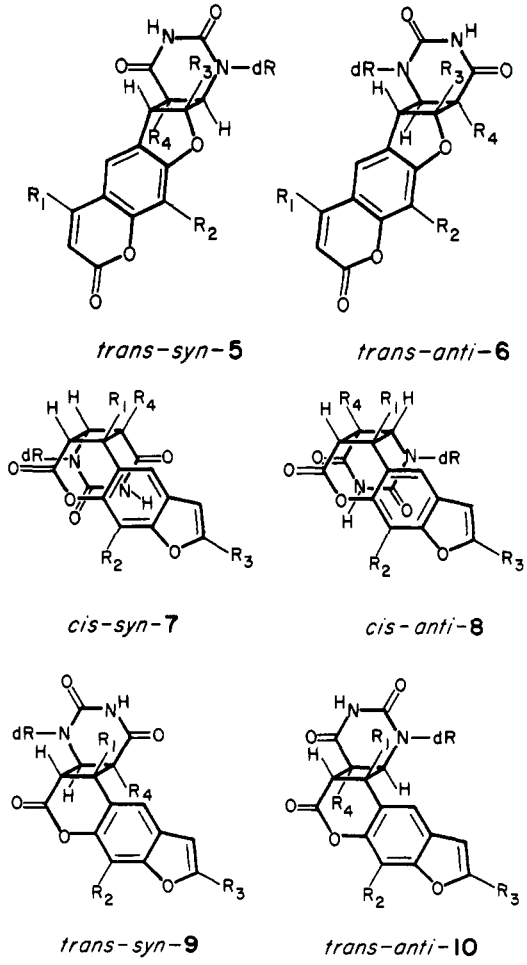


*cis-syn-3* *cis-syn-3'* *cis-syn-4*

stereochemistry was obtained from NOE enhancement measurements on the aglycon derivative of F37 and F38, 8-MOP-F40H$^+$ (Table IV).

The $^1$H NMR data obtained on F36 are summarized in Table III. These data are consistent with its structural assignment as a furan-side psoralen–deoxyuridine adduct, having cis–syn stereochemistry. In this case there are four protons about the cyclobutane ring, resulting in four multiplets. There are two sets of approximately equal coupling constants, with $J_{4',5'} = J_{4',5} = 5.5$ Hz and $J_{5',6} = J_{5,6} = 9.2$ Hz. The small quantities of F36 available for analysis made it difficult to accurately measure the values of the $^4J$ cross couplings, although an upper limit of 1.5 Hz was established. As was found for F37 and F38, there is an additional complication due to coupling between the 4'-H and 5-H of the psoralen. There was insufficient material to attempt NOE enhancement experiments on this adduct.

The $^1$H NMR spectrum of 8-MOP-F41 confirmed its assignment as a pyrone-side monoadduct to thymidine (Table III and Figure 5). The psoralen protons at 4' and 5' are present in the aromatic region, while the 3-H and 4-H have undergone upfield shifts into the 3.4–4.5-ppm region. The 3-H is coupled to both 4-H and 6-H of thymidine, resulting in a doublet of doublets at 3.56 ppm. The two cyclobutyl doublets collapse to singlets when the 3-H is irradiated. The regiochemistry of this system is therefore syn, as shown in structures **7** and **9**. The signal to noise ratio for this sample was not high



*trans-syn-5* *trans-anti-6*

*cis-syn-7* *cis-anti-8*

*trans-syn-9* *trans-anti-10*

enough to obtain reliable NOE enhancements. While this does not allow an unambiguous assignment of cis or trans stereochemistry, the cis configuration (structure **7**) is most consistent with the proposed model for psoralen–DNA interactions

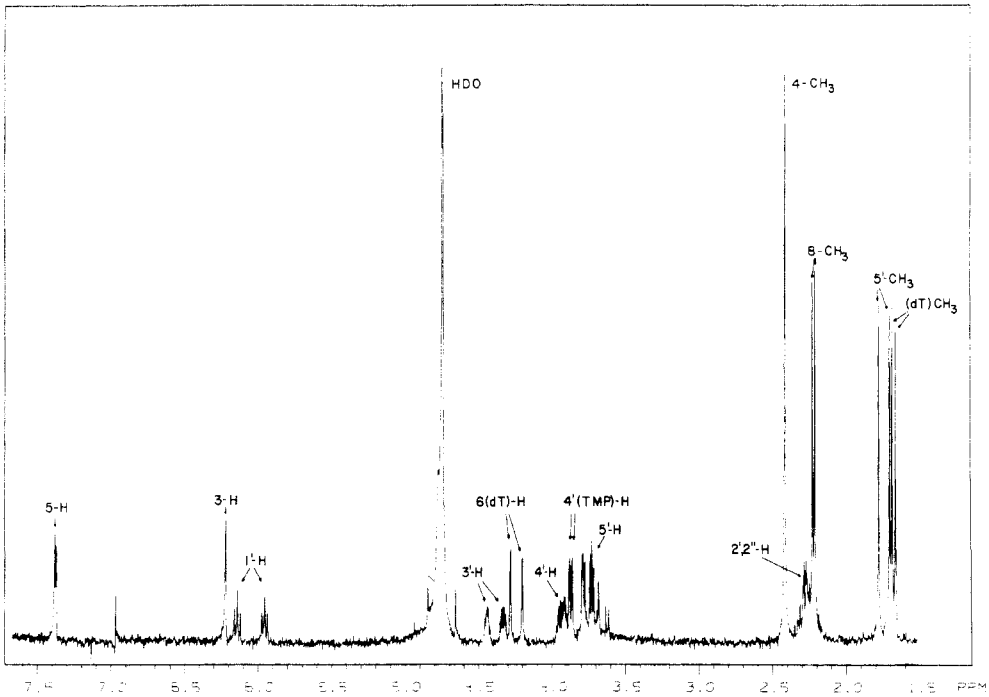



FIGURE 6: 360-MHz $^1$H NMR spectrum of TMP-F49.

(Straub et al., 1981). The $^1$H NMR spectrum of 8-MOP-F42H$^+$ (Table III) is consistent with its assignment as an aglycon derivative of 8-MOP-F41.

(*2*) *TMP–DNA Adducts.* The $^1$H NMR spectrum of TMP-F49 is shown in Figure 6. Obviously there are more resonances present in this spectrum than are required by a single TMP–nucleoside adduct. The mass spectral results clearly indicate the presence of a TMP–thymidine adduct. Most of the proton and methyl resonances occur in pairs with slight chemical shift differences, suggesting the presence of two diastereomers in approximately a 1:1 ratio. The 4′-H and 5′-$CH_3$ resonances of the TMP moiety along with the 5-$CH_3$ and 6-H resonances of the thymidine are shifted to higher field, indicating that cycloaddition has occurred on the furan side of the psoralen.

No vicinal coupling of the two cyclobutane protons was observed for either diastereomer, reflecting a syn orientation. There is cross coupling ($J_{4',6} \sim 2$ Hz) of the diagonally positioned 6-H (dT) and 4′-H (TMP), analogous to the situation observed for the 8-MOP–thymidine adducts. In order to clearly reveal the cross coupling between the two cyclobutane protons, it was necessary to decouple the 5-H of the TMP moiety. Although there was insufficient TMP-F49 to provide the signal to noise ratio necessary for NOE enhancement measurements, enough of the aglycon derivative of F49 for NOE analysis was obtained by acid hydrolysis of a sample of TMP-modified DNA (TMP-F50H$^+$). Irradiation of either of the two cyclobutyl methyls [5′-$CH_3$ and 5-$CH_3$ (dT)] showed relatively large and approximately equal enhancements for both of the cyclobutyl protons (Table V). This result is consistent only with a structure where both methyls are cis and adjacent to both cyclobutyl protons. The aglycon of TMP-F49 (and therefore F49) thus has the same cis–syn stereochemistry as found for the 8-MOP–nucleoside adducts.

TMP-F47 had an $^1$H NMR spectrum consistent with its assignment as a TMP–deoxyuridine adduct. There are three methyl resonances, corresponding to the $CH_3$'s at C-4, C-8, and C-5′ of TMP. The cyclobutyl system consists of three protons and the 5′-$CH_3$ from the TMP moiety. The 5-H resonance of the deoxyuridine is an apparent triplet, indicating that $J_{4',5} = J_{5,6}$. Irradiation at 5-H of the deoxyuridine results in a pair of apparent singlets for 4′-H of the TMP and 6-H of the pyrimidine residue; this implies that cross coupling between these diagonally positioned protons is less than the 1.5-Hz line width of the signals. These results are consistent with cis–syn stereochemistry. In addition, F47 as isolated appears to be a single diastereomer.

## Discussion

The photocycloaddition of a pyrimidine to a psoralen generates a cyclobutane ring with four asymmetric centers. In nucleoside adducts, an additional invariant chiral center is present in the deoxyribose moiety. Reaction can take place at either the 4′,5′-furan- or 3,4-pyrone-side double bonds of the psoralen. This addition can occur in either a *syn* or *anti* configuration, where *syn* refers to a configuration where the 5 position of the pyrimidine and the 5′ or 3 position of the psoralen are at diagonally opposed corners of the cyclobutane ring. *Anti*, then, refers to a configuration where the 5 position of the pyrimidine is adjacent to either the 5′ or 3 position of the psoralen. There are thus four sets of diastereomers (furan-side syn, furan-side anti, pyrone-side syn, and pyrone-side anti), each having in theory $2^4 = 16$ possible isomers. Twelve of the sixteen isomers will incorporate a trans-fused cyclobutane. While such a trans-fused four-membered ring is possible, the steric strain present in a tricyclo[4.0.0.3] system incorporating one or two trigonal centers would be prohibitive. As discussed above, NOE enhancement measurements are an effective tool for unequivocally assigning stereochemistry in such systems.

The four remaining isomers which incorporate a cis-fused cyclobutane are in fact two pairs of enantiomers if one disregards the presence of the deoxyribose. This can occur at each of the possible addition sites on the psoralen, so that there are a total of eight possible pairs of enantiomers. The notation cis or trans is used to refer to whether the pyrimidine and psoralen moieties are on the same or opposite sides of the cyclobutane ring. These eight possible isomers are denoted as structures **3–10**. Each structure has an accompanying diastereomer, where the configuration at each asymmetric

Table V: Chemical Shifts ($\delta$)[a] and Coupling Constants of TMP-DNA Monoadducts

| moiety[b] | F47 | F49 (49 + 49′) | F50H$^+$ |
|---|---|---|---|
| C-5 H (TMP) | 7.378 s | 7.379 s | 7.350 s |
| C-3 H (TMP) | 6.249 s | 6.233 s | 6.279 s |
| C-1′ H (dR) | 6.134 t | 6.144, 5.954 t | |
| C-6 H (U) | 4.560 m, $J_{5,6}$ = 9.2 Hz, $J_{4',6}$ = 1.5 Hz | | |
| C-3′ H (dR) | 4.443 m | 4.450, 4.340 m | |
| C-6 H (T) | | 4.281, 4.200 d, $J_{4',6}$ = 2 Hz | 3.824 d, $J_{4',6}$ = 2 Hz |
| C-4′ H (TMP) | 4.285 m, $J_{4',5}$ = 9.2 Hz, $J_{4',6}$ = 1.5 Hz | 3.880, 3.861 dd, $J_{4',6}$ = 2 Hz, $J_{4',5(TMP)}$ = 1 Hz | 3.796 dd, $J_{4',6}$ = 2 Hz, $J_{4',5(TMP)}$ = 1 Hz |
| C-5 H (U) | 4.041 t, $J_{5,6} = J_{4',5}$ = 9.2 Hz | | |
| C-4′ H (dR) | 3.922 m | 3.920 m | |
| C-5′ H (dR) | 3.722 m | 3.750 m | |
| C-4 $CH_3$ (TMP) | 2.432 s | 2.410 s | 2.427 s |
| C-2′,2″ $CH_3$ (dR) | 2.287 m | 2.280 m | |
| C-8 $CH_3$ (TMP) | 2.246 s | 2.226, 2.208 s | 2.244 s |
| C-5′ $CH_3$ (TMP) | 1.771 s | 1.775, 1.702 s | 1.703 s |
| C-5 $CH_3$ (T) | | 1.685, 1.602 s | 1.684 s |

[a] $\delta_{HDO}$ = 4.75. [a] TMP = 4,5′,8-trimethylpsoralen; dR = deoxyribose; U = uracil; T = thymine.

center on the cyclobutane ring is opposite that shown. The presence of an additional invariant asymmetric center in the deoxyribose moiety of nucleoside adducts yields a pair of diastereomers rather than enantiomers. Removal of the deoxyribose will result in pairs of enantiomers. The relationship between these diastereomeric pairs is illustrated for the cis–syn isomers, structures **3** and **3′**.

The mass spectral and $^1$H NMR results outlined above indicate that both 8-MOP and TMP undergo photoreaction with DNA to yield as major products a pair of diastereomeric furan-side thymidine adducts. The two psoralens also undergo reaction with deoxycytidine (and perhaps 5-methyldeoxycytidine) residues. The NMR results unequivocally define the stereochemistry of these adducts as cis–syn furan-side adducts, where cycloaddition has occurred between the 4′,5′ double bond of the psoralen and the 5,6 double bond of the pyrimidine. The pairs of 8-MOP–thymidine (F37 and F38) and TMP–thymidine (F49) adducts therefore have the stereochemical relationship represented in structures **3** and **3′**. That is, the stereochemistry at each symmetrical center of the cyclobutane ring is opposite and equal, but the presence of an invariant chiral center in the deoxyribose results in a pair of diastereomers.

Removal of the deoxyribose can be accomplished by acid hydrolysis, since reduction of the 5,6 double bond in pyrimidine nucleosides greatly increases the acid lability of the C–N glycosidic bond. When acid hydrolysis is carried out on 8-MOP-F37 and -F38, they are converted to thymine–8-MOP adducts which coelute under our standard HPLC conditions. The two hydrolysis products have opposite and equal circular dichroism spectra, as expected for a pair of enantiomers. The CD spectra of F37 and F38 are also approximately opposite and equal (Figure 7), since the deoxyribose moiety influences the overall molar ellipticity of each diastereomer to a small degree. Similar results were obtained for TMP-F49. The $^1$H NMR spectrum of TMP-F49 indicates that it is actually a 1:1 mixture of diastereomeric adducts, each having cis–syn stereochemistry. Acid hydrolysis of this mixture results in apparently a single product by NMR, as expected for an enantiomeric mixture.

The formation of diastereomeric pairs of adducts can be explained by considering the steps involved in the binding of psoralens to DNA. The formation of a noncovalent complex occurs by intercalation of the psoralen between adjacent pairs of the DNA double helix (Wiesehahn & Hearst, 1978). This complex can then undergo photoreaction to yield a covalent

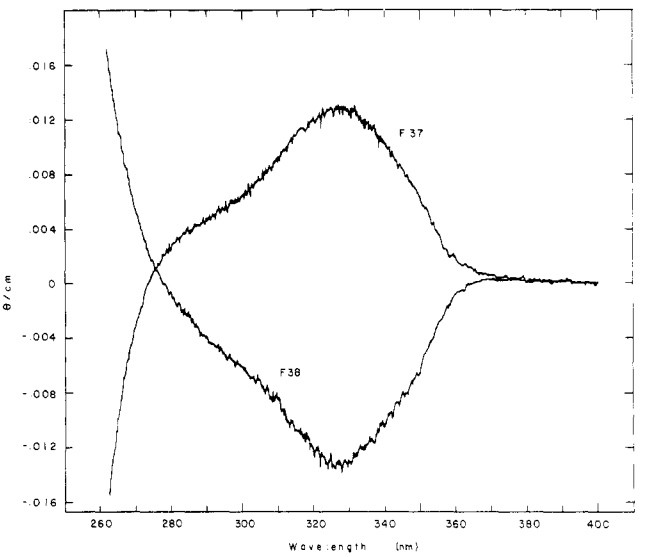



FIGURE 7: Circular dichroism spectra of 8-MOP-F37 and 8-MOP-F38.

nucleoside–psoralen adduct. The question of which of the two possible cis–syn diastereomers is formed is determined by whether the psoralen is on top or underneath of a given base pair.

The deoxyuridine adducts found for the two psoralens (8-MOP-F36 and TMP-F47) appear to be single cis–syn diastereomers based on their $^1$H NMR. It is possible that their accompanying diastereomers are present as minor uncharacterized products in our chromatographic system. Also, 8-MOP-F41 may consist of more than a single diastereomer, but complete analysis of this fraction is complicated by the facile hydrolysis of the lactone in 3,4-dihydropsoralen derivatives, leading to multiple products. An accurate measurement of the relative amounts of the two diastereomeric adducts (8-MOP-F37 and -F38) formed in the reaction with double-stranded DNA is important, since a preference for the formation of one diastereomer indicates a preference by the psoralen for intercalation on top, or underneath, of an AT base pair. Such short-range sequence specificity has been observed with other intercalating agents such as ethidium bromide (Kastrup et al., 1978).

The results in Table I indicate a preference in the diastereomeric ratio of F38:F37 of 7:4. Confirmation of this preference has been obtained by hydrolyzing 8-MOP-modified DNA and measuring the CD of the isolated 8-MOP–thymi-

dine adduct, 8-MOP-F40H$^+$. The CD was compared to the CD spectrum of the pure enantiomer obtained by acid hydrolysis of 8-MOP-F37 and -F38. In contrast, the two TMP–thymidine adducts present in TMP-F49 appear to be formed in a 1:1 ratio. The absolute configurations of these adducts are currently under investigation and will allow us to assign the sequence specificity of 8-MOP as (5′)TpX or (5′)XpT.

The results obtained in this study indicate that complete hydrolysis of psoralen-modified DNA can be achieved, either enzymatically or by treatment with mild acid. Characterization of the psoralen-modified DNA by HPLC will then allow direct quantification of the number and type of psoralen–nucleoside monoadducts and cross-links present in the DNA. This methodology has the potential of becoming a standard assay for the detection of both mono- and diadducts in psoralen-modified nucleic acids.

The results of the present study are similar to those obtained previously by us with a synthetic psoralen derivative, 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT) (Straub et al., 1981). The photoreaction of HMT with double-stranded DNA yields a set of psoralen–nucleoside monoadducts similar to those observed for TMP. The major products are diastereomeric cis–syn, furan-side adducts with thymidine and deoxyuridine (deoxycytidine) residues. With both HMT and TMP, addition to the 3,4 (pyrone) double bond of the psoralen is a minor reaction compared to addition to the 4′,5′ (furan) double bond. In contrast, the reaction of 8-MOP with DNA results in a substantial amount (19%) of the pyrone-side adduct, F41. [We have found that reirradiation of the isolated nucleoside–psoralen–nucleoside diadduct (8-MOP-F32) at 365 nm for 1 h does not result in photoreversion to monoadducts]. These results do not agree with predictions of the sites of reactivity in psoralens made by others (Song et al., 1971; Chatterjee & Cantor, 1978).

A possible explanation for the observed specificity is that the steric constraints imposed by the noncovalent psoralen–DNA intercalation complex prior to irradiation in part determines the stereochemistry of the products. A model for such an intercalation complex has been proposed, where photoreaction with adjacent pyrimidine residues will lead to the observed furan- and pyrone-side, cis–syn stereochemistry at either the 4′,5′ or 3,4 double bonds of the psoralen. It is possible that a methyl substituent at either the 3 or 4 position of the psoralen leads to unfavorable steric interactions with the 5-methyl of an adjacent thymine, resulting in a decreased yield of pyrone-side adducts. This interaction is absent for 8-MOP and could account for the increased yield of pyrone-side adduct observed with this psoralen. Other factors such as electronic effects and solubility considerations will of course influence the overall quantum yield of the psoralen–DNA reaction. This model also predicts that cis–syn stereochemistry is in part the result of carrying out the photoreaction with native, double-stranded DNA. It is possible that other diastereomeric adducts could result from reaction with alternative nucleic acid secondary or tertiary structures. Should this be the case, the psoralens could be uniquely useful as probes of secondary and tertiary nucleic acid structure. Experiments are currently under way to test this hypothesis.

## Conclusions

We have isolated and characterized four mononucleoside adducts from the photoreaction of 8-MOP or TMP with native, double-stranded DNA. Two diastereomeric adducts of thymidine are formed by cycloaddition to the 4′,5′ (furan) double bond of the psoralen, with cis–syn stereochemistry. Small amounts of a cis–syn deoxycytidine adduct are also formed, which undergoes hydrolytic deamination to a deoxyuridine product. In the case of 8-MOP, substantial quantities of a pyrone-side adduct are formed, where cycloaddition has taken place at the 3,4 double bond of the psoralen. The photoreaction between TMP and DNA results in only minor quantities of a thymidine–pyrone-side adduct. All of the adducts have cis–syn stereochemistry, determined in part by the constraints imposed by the DNA helix on the geometry of the noncovalent intercalation complex formed by the psoralen and DNA prior to irradiation. Acid hydrolysis studies on 8-MOP- or TMP-modified DNA resulted in the isolation and characterization of a mixture of enantiomeric cis–syn thymine–psoralen adducts, identical with the aglycon derivatives of the thymidine adducts. With double-stranded DNA there is a 7:4 preference for the formation of one diastereomeric nucleoside–8-MOP adduct, indicating a specificity by the psoralen for a (5′)TpX or (5′)XpT sequence. The methodology described in this study provides an assay for the complete characterization of both mono- and diadducts in psoralen-modified nucleic acids.

## Acknowledgments

The authors thank Dr. J. Dallas and A. Ribiero for their assistance in obtaining NMR data.

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# Purification and Properties of an Aminoglycoside Acetyltransferase from *Pseudomonas aeruginosa*†

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ABSTRACT: An aminoglycoside 3-acetyltransferase [AAC(3)], possibly a new isoenzymic species of the 3-*N*-acetyltransferase group, was purified to apparent homogeneity from a crude extract of *Pseudomonas aeruginosa*, a gentamicin-resistant clinical isolate. The method of purification was consecutive column chromatography—(i) gel filtration, (ii) affinity chromatography, and (iii) ion-exchange chromatography—to give two protein peaks, one of which was coincident with activity and which indicated a purification of 600 (specific activity = 9.743 units $mg^{-1}$ at pH 7.2, 34 °C). Polyacrylamide disc gel electrophoresis indicated a single protein band coincident with enzymic activity. The molecular weight of the enzyme was about 39000. AAC(3)-V (provisonal designation) was further characterized by stability, substrate, pH, and kinetic studies. The $K_m$ was 0.724 μM (sisomicin), and the $V_{max}$ was 0.102 μmol $min^{-1}$ $mg^{-1}$ (sisomicin) at pH 7.2 and 34 °C. Substrate inhibition was exhibited by kanamycin A and tobramycin. Studies showed that enzyme activity was significantly stabilized when preparations contained substrate.

The major cause of aminoglycoside resistance in bacteria is now recognized as aminoglycoside-modifying enzymes (Davies & Smith, 1978; Devaud et al., 1977). Aminoglycoside antibiotics are inactivated by three enzyme-catalyzed transferase reactions—O-adenylylation, O-phosphorylation, and N-acetylation (Benveniste & Davies, 1973). *N*-Acetyltransferases modify aminoglycosides at the 6′- and 2′-amino positions of ring I (purpurosamine in gentamicin) and at the 3-amino position of ring II (deoxystreptamine). N-Acetylation on ring III (3-aminoglucose in kanamycin, tobramycin, and amikacin) is not known to occur (Figure 1). Hence, there are three acetyltransferase enzymes according to a classification indicated by the position of modification on the drug molecule (Haas & Dowding, 1975). In addition, there are subgroups of isoenzymes which modify the antibiotics at the same position but differ in their substrate profiles and in other biochemical properties. Thus, at present there are four isoenzymic forms of aminoglycoside 3-acetyltransferase (Davies & Smith, 1978).

This paper describes a chromatographic scheme for the purification of aminoglycoside 3-acetyltransferase from a crude extract of *Pseudomonas aeruginosa*, a clinical isolate resisant to kanamycin, gentamicin, tobramycin, and sisomicin. The method resulated in a 460-fold purification and gave a 23% recovery of the enzyme. Full characterization of the enzyme established that it was a new isoenzymic member of the 3-*N*-acetyltransferase group.

The degree of purity reported in this work is significant since many attempts to purify modifying enzymes have been unsuccessful, with one notable exception (Williams & Northrop, 1976), and have resulted in partial purifications only. The recovery of AAC(3)-V in high purity and activity was beneficial for kinetic and other studies, and for low background spectrophotometric assays, including serum gentamicin assay.

## Materials and Methods

A gentamicin-resistant *Pseudomonas aeruginosa* culture was obtained as a clinical isolate from Sydney Hospital (July, 1977) after isolation with tryptone soy agar plates at 37 °C. Tryptone soy agar and broth were from Oxoid. CNBr–Sepharose 4B, Sephacryl S-200, and DEAE-Sephacel were obtained from Pharmacia. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), acetyl coenzyme A, kanamycin, and neomycin were purchased from Boehringer Mannheim. Low molecular weight calibration proteins for gel filtration were obtained from Sigma. Media for polyacrylamide disc gel electrophoresis was purchased from Eastman Organic Chemicals. Sisomicin and gentamicin were gifts from Schering Corp. (Essex Laboratories, Australia). Amikacin was a gift from Bristol-Myers (Australia), and tobramycin was a gift from Eli Lilly (Australia). All other reagents were of analytical grade.

*Separation of the Kanamycin Components.* Kanamycins A and B were separated from commercial kanamycin sulfate by the method of Inouye & Ogawa (1964). Kanamycins A and B were obtained as freeze-dried white translucent crystalline solids. The homogeneity of each component was established by paper chromatography.

*Paper Chromatography of Kanamycins A and B.* A descending paper chromatogram (Whatman no. 2 paper) was developed as described by Mason et al (1961). Kanamycin A ($R_f$ 0.16–0.22) and kanamycin B ($R_f$ 0.24–0.38) were de-

† From the Department of Pharmacy, University of Sydney, Sydney, Australia 2006. *Received January 22, 1981.* This work was supported in part by a grant from the National Health and Medical Research Council.

0006-2960/82/0421-0871$01.25/0 