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Formation of Cyclobutane Thymine Dimers Photosensitized by Pyridopsoralens: Quantitative and Qualitative Distribution within DNA†

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ABSTRACT: As after irradiation with 254-nm UV light, exposure of thymidine and three isomeric pyridopsoralen derivatives to UVA radiation, in the dry state, leads to the formation of the six diastereomers of cyclobutadithymidine as the predominant reaction. This unexpected photosensitized reaction, which also gives rise to both 5*R** and 5*S** diastereomers of 5,6-dihydro-5-(α-thymidylyl)thymidine (or "spore" photoproduct), is selective since [2 + 2] dimerization of 2′-deoxycytidine was not detected under the same experimental conditions. The cis-syn isomer of cyclobutadithymine was also found to be produced within isolated DNA following UVA irradiation in aqueous solutions containing 7-methylpyrido[3,4-*c*]psoralen. Quantitatively, this photoproduct represents about one-fifth of the overall yield of the furan-side pyridopsoralen [2 + 2] photocycloadducts to thymine. DNA sequencing methodology was used to demonstrate that pyridopsoralen-photosensitized DNA is a substrate for T4 endonuclease V and *Escherichia coli* photoreactivating enzyme, two enzymes acting specifically on cyclobutane pyrimidine dimers. Furthermore, the dimerization reaction of thymine is sequence dependent, with a different specificity from that mediated by far-UV irradiation as inferred from gel sequencing experiments. Interestingly, adjacent thymine residues are excellent targets for 7-methylpyrido[3,4-*c*]psoralen-mediated formation of cyclobutadithymine in TTTTA and TTAAT sites, which are also the strongest sites for photoaddition. The formation of cyclobutane thymine dimers concomitant to that of thymine–furocoumarin photoadducts and their eventual implication in the photobiological effects of the pyridopsoralens are discussed.

Psoralens are natural or synthetic photosensitizers used in photochemotherapy of various skin diseases including psoriasis, mycosis fungoides, and vitiligo (Knobler et al., 1988). The therapeutic effects of these agents are likely to result, at least partly, from the induction of DNA photoadducts, although other cellular components including proteins and lipids may also be considered as important targets (Laskin et al., 1986; Midden, 1988; Averbeck, 1989; Cadet et al., 1990). The mutagenic and carcinogenic effects associated with the photobiological action of psoralens are mostly explained in terms of DNA photodamage (Ben-Hur & Song, 1984; Averbeck, 1989). The major products resulting from the photochemical reactions of psoralens with DNA are mono- and bicycloaddition products involving mostly the thymine moiety (Hearst et al., 1984; Vigny et al., 1985). DNA photosensitization by certain furocoumarins, such as 3-carbethoxypsoralen, may also involve oxygen and result in photooxidation of guanine residues (Cadet et al., 1984; Sage et al., 1989).

The three synthesized monofunctional pyridopsoralens (Moron et al., 1983) (Figure 1) present interesting potentiality for the phototreatment of psoriasis (Dubertret et al., 1985). The location of the nitrogen atom within the pyridine ring was found to play a major role in the photobiological action of the three isomeric pyridopsoralens since 7-methylpyrido[3,4-*c*]-psoralen (MePyPs)[1] is more lethal and mutagenic than the

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[1] Abbreviations: PyPs, pyrido[3,4-*c*]psoralen; MePyPs, 7-methylpyrido[3,4-*c*]psoralen; 2N-MePyPs, 7-methylpyrido[4,3-*c*]psoralen; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; ^{252}Cf-PDMS, ^{252}Cf plasma desorption mass spectrometry; FABMS, fast atom bombardment mass spectroscopy; NMR, nuclear magnetic resonance spectroscopy; t_R, retention time; UVA, ultraviolet radiation of class A (320–400 nm); UVC, ultraviolet radiation of class C (220–280 nm); Pyr<>Pyr, cyclobutadipyrimidine or cyclobutane pyrimidine dimer; dThd, thymidine; dThd<>dThd, cyclobutadithymidine; Thy<>Thy, cyclobutadithymine; Pso<>dThd, psoralen monophotocycloadduct to thymidine; dThd(α–5)dThd, 5,6-dihydro-5-(α-thymidylyl)thymidine; bp base pair(s); PRE, photoreactivating enzyme; dNTP, deoxyribonucleotide triphosphates; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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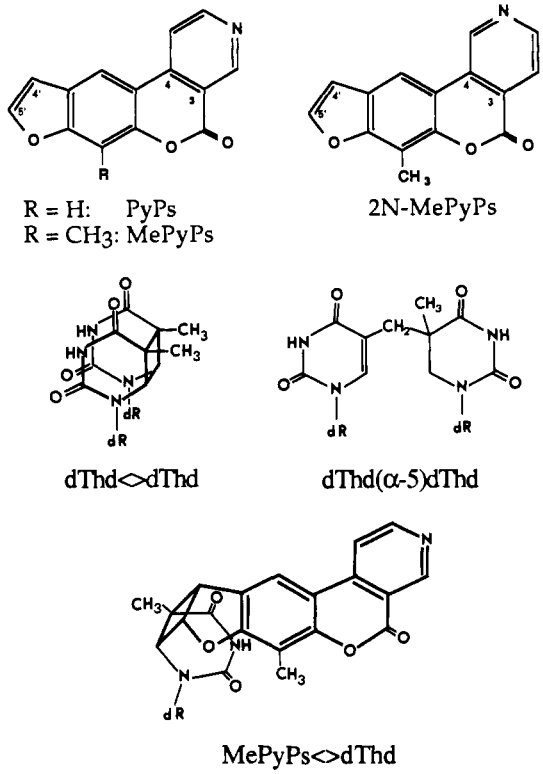

FIGURE 1: Molecular structures of pyrido[3-4-*c*]psoralen (PyPs), 7-methylpyrido[3,4-*c*]psoralen (MePyPs), 7-methylpyrido[4,3-*c*]-psoralen (2N-MePyPs), *cis-syn*-cyclobutadithymidine (dThd<>dThd), 5,6-dihydro-5-(α-thymidylyl)thymidine (dThd(α-5)dThd), and the cis-syn furan-side MePyPs monocyclophotoadduct to thymidine (MePyPs<>dThd).

isomeric 7-methylpyrido[4,3-*c*]psoralen (2N-MePyPs) (Blais et al., 1984, 1987). However, structural studies involving isolated DNA demonstrate that the two compounds induce the same type of photoadducts (Moysan, 1987; A.M., unpublished data). Although pyridopsoralens are monofunctional compounds, they are more cytotoxic than the bifunctional compound 8-methoxypsoralen (8-MOP) in both yeast and mammalian cells (Moustacchi et al., 1983; Averbeck, 1985; Papadopoulo et al., 1986; Blais et al., 1987). Moreover, pyridopsoralens were found to be equally or more mutagenic and recombinogenic in lower and higher eucaryotes (Billardon et al., 1984; Papadopoulo et al., 1986; Blais et al., 1987). This may be related to the accumulation of single-strand breaks following treatment of either yeast (Magana-Schwencke & Moustacchi, 1985) or human cells (Nocentini, 1986) with pyridopsoralen as opposed to 8-MOP. Although a high photobinding capacity of pyridopsoralens was found both in vitro (Blais et al., 1984) and in vivo (Averbeck, 1985; Magana-Schwencke & Moustacchi, 1985; Nocentini, 1986), the photoreactive sites for pyridopsoralens, as a function of the sequence context, did not differ from that observed for other psoralen derivatives (Boyer et al., 1988). Taken together, all these observations do not entirely account for the high photobiological activity of pyridopsoralens. This is why the existence of other photolesions induced by pyridopsoralens could have been suspected.

It was recently shown that these pyridopsoralens are able to photoinduce the cyclodimerization of thymine through a triplet energy-transfer mechanism (Costalat et al., 1990). Further information on the new pyridopsoralen-mediated photoreaction of the thymine moiety is provided in the present study by using thymidine and DNA as the substrates. Near-UV irradiation of a mixture of thymidine and each of the three pyridopsoralens in the dry state was found to generate isomeric cyclobutadithymidines and 5,6-dihydro-5-(α-thymidylyl)thymidines in addition to the expected pyrimidine–psoralen [2 + 2] photocycloadducts (Figure 1). Thymine photodimerization was also found to occur within DNA exposed to UVA radiation in the presence of MePyPs. Sequence effects on the MePyPs-mediated formation of cyclobutadithymine are presented and compared with those induced by 254-nm UV light.

Experimental Procedures

(*1*) *Chemicals.* Formic acid (98%) was from Sigma (St. Louis, MO). The psoralen derivatives including 7-methylpyrido[3,4-*c*]psoralen (MePyPs), pyrido[4,3-*c*]psoralen (PyPs), and 7-methylpyrido[4,3-*c*]psoralen (2N-MePyPs) were synthesized by Drs J. Moron and E. Bisagni at Institut Curie, Orsay (Moron et al., 1983). Thymine and thymidine, which were obtained respectively from Sigma and Genofit (Geneva, Switzerland), were utilized without any further purification. 2′-Deoxycytidine was from Fluka (Buchs, Switzerland). *cis-syn*-Cyclobutadithymine was prepared by far-UV irradiation of frozen aqueous solution of thymine and purified by crystallization from the thawed aqueous solution (Wang, 1961). The purity was found to be higher than 99% as determined by reversed-phase high-performance liquid chromatography (HPLC) (Cadet et al., 1980). The six diastereoisomers of dThd<>dThd were prepared by acetone-mediated photosensitization and purified by HPLC with use of both silica gel and octadecyl-bonded columns (Cadet et al., 1985). [C^3H_3] and [$^3H_6$]thymidine with a specific activity of 48 Ci/mmol and 20 Ci/mmol, respectively, were from Amersham (U.K.). [$^{14}CH_3$]Thymidine, which was purchased from the Departement des Radioelements, CEN Saclay, France, was purified on an analytical Nucleosil ODS column (Macherey-Nagel, Dären, FRG) with use of water/methanol (9:1 v/v) to remove self-irradiation decomposition products. [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) was obtained from New England Nuclear. "Ready-solv HP/b" scintillating liquid was purchased from Beckman (Fullerton, CA).

(*2*) *Biochemicals.* λ phage DNA (New England Biolabs, Taunus, FRG) was labeled with [C^3H_3]- and [$^3H_6$]thymidine by nick translation (nick-translation kit, Boehringer, Manheim, FRG) and purified on a GP50 nick column from Pharmacia (Uppsala, Sweden) prior to use. DNase I and acidic phosphatase were from Sigma, whereas S_1 nuclease was purchased from Pharmacia. Restriction enzymes, T4 polynucleotide kinase, and T4 DNA polymerase were from Bethesda Research Laboratories (Bethesda, MD). T4 endonuclease V purified in the laboratory of Dr. P. Hanawalt (Stanford University, Stanford, CA) and *E. coli* DNA photolyase prepared in the laboratory of Dr. A. Sancar (University of North Carolina, Chapel Hill, NC) were a gift from Dr. A. Sarasin (Villejuif, France). 5′-End-labeled DNA fragment of 123-bp length, derived from *Eco*RI plus *Pvu*II restriction of phage M13mp8 RF DNA, was purified as previously described (Sage & Moustacchi, 1987).

(*3*) *Chromatographic Analysis.* The high-performance liquid chromatographic equipement used for the quantitative measurement of *cis-syn*-cyclobutadithymine was a Model 6000 dual piston pump from Waters Associates (Mildford, MA). The detection of *cis-syn*-cyclobutadithymine was monitored at 232 nm with use of a Cecil Model CE 212 variable wavelength spectrometer (Cecil Instruments, Cambridge, U.K.). The separation was achieved on an ODS-2 column. (25 × 0.46 cm i.d.; mean particle size 5 μm) from Whatman (Clifton, NJ) equipped with an anionic pellicular precolumn. The HPLC

system used for analysis as MePyPs–thymidine photocycloadducts within DNA consisted of a Beckman Model 344 pump equiped with a Kontron Model SFM 25 fluorometer (λ_{exc} = 340 nm, λ_{em} = 430 nm). The column was a Lichrospher 100 RP-18 (Merck, Darmstadt, FRG) (25 × 0.46 cm i.d.; mean particle size 5 μm) eluted with water/methanol (60:40 v/v) at a flow rate of 1 mL min^{-1} and at a temperature of 25 °C.

Two-dimensional TLC separations were carried out on precoated silica gel plastic plates (20 × 20 cm), F 254 from Merck. Eluent 1 consisted of the lower phase of the ternary system chloroform/methanol/water (4:2:1) to which 5% methanol was added (Cadet et al., 1978). Eluent 2 was a mixture of ethyl acetate, propan-2-ol, and water (75:16:9). The visualization of the spots containing *cis-syn*-cyclobutadithymine was performed by fluorescence quenching, subsequent to photoreversion of Thy<>Thy into thymine with a 254-nm emitting Desaga mineral lamp. Quantitative determination was made by scraping off the silica gel containing the radiolabeled compounds and counting for radioactivity after the addition of 3 mL of scintillation liquid.

(*4*) *Near-UV Irradiation of Thymidine and Pyridopsoralens in the Dry State.* A mixture of pyridopsoralen derivative and thymidine (relative ratio 1:10, w/w) in methanol was evaporated to dryness in petri dishes, giving rise to a thin film. These samples were irradiated at 360 nm according to the procedure previously described (Cadet et al., 1983a), with an array of six black light lamps, Model 08RSF 40 BLB (Philips, Holland)

(*5*) *UVA Irradiation of Isolated DNA in the Presence of MePyPs.* Dark complex was prepared by the addition of 40 μL of an ethanolic solution of MePyPs ($c = 4.6 \times 10^{-6}$ M) to 1 mL of [^{3}H]thymidine phage DNA ($c = 1.33 \times 10^{-3}$ M, $a = 7.7 \times 10^{13}$ cpm/mol) in 10 mM Tris-HCl/1 mM EDTA (pH 8) buffer. This solution was gently stirred during an hour for the formation of the complex. Irradiation of this complex was performed at 365 nm using a HPW 125 Philips mercury lamp equipped with a pyrex glass water filter until the absorbed dose was 0.93 kJ m^{-2}. The bandwidth was 8 nm and the dose rate 15 J m^{-2} s^{-1}. The sample of photomodified DNA was divided into two fractions (one-third for the determination of the rate of photoaddition products, two-thirds for the measurement of *cis-syn*-cyclobutadithymine) and precipitated by addition of cold ethanol.

(*6*) *Photoreaction of MePyPs with 5′-^{32}P-End-Labeled DNA.* A total of 1 μL of MePyPs solution in dimethyl sulfoxide was added at a final concentration of 2.5×10^{-4} M to 19 μL of a solution of labeled DNA in TE buffer (10 mM Tris-HCl, pH 8.0/1 mM EDTA), and the solution was left in the dark for 30 min at room temperature. The reaction mixture was irradiated with UVA light at a fluence of 54 or 18 kJ m^{-2} as previously described (Sage & Moustacchi, 1987). After irradiation, the unbound material was eliminated by extraction with chloroform/isoamyl alcohol (19:1 v/v) followed by ethanol precipitation of DNA.

(*7*) *UVC Irradiation of 5′-^{32}P-End-Labeled DNA.* ^{32}P-Labeled DNA dissolved in TE buffer was exposed on ice to UVC irradiation of a germicidal lamp (mainly 254 nm). The total fluence delivered was 2 kJ m^{-2} with an intensity of 7.5 J m^{-2} s^{-1} as measured with a Latarjet dosimeter (Institut Curie, Paris).

(*8*) *Quantitative Measurement of MePyPs Photocycloadducts to Thymidine Generated within Isolated DNA.* Enzymatic hydrolysis of the DNA for the determination of the photoaddition products of MePyPs to thymidine was achieved by using DNase I, S_1 nuclease, and acidic phosphatase in acetate buffer (pH 4.6) according to a procedure already described (Moysan et al., 1988).

(*9*) *Determination of the cis-syn-Cyclobutadithymine within DNA Exposed to Near-UV Radiation in the Presence of MePyPs.* Treated DNA was dissolved in formic acid (98%), and the resulting solution was heated at 180 °C for 90 min in thick sealed tube (Carrier & Setlow, 1971; Cadet et al., 1983b). Then, the solution was cooled and subsequently evaporated to dryness. The resulting residue was dissolved in water containing authentic *cis-syn*-cyclobutadithymine as a cold marker and deposited on the top of the analytical ODS-2 column (Cadet et al., 1983b). The column was eluted with distilled water (pH 6.0) at a flow rate of 1 mL/min. Fractions were collected every minute in 5-mL Zinssen analytical tubes and subsequently counted for radioactivity after the addition of 3 mL of scintillation liquid.

(*10*) *Photoreversion of cis-syn-Cyclobutadithymine.* Far-UV mediated reversion of the main thymine photoproduct isolated by HPLC was performed in aqueous solution. Typically cold authentic *cis-syn*-cyclobutadithymine was added to an aqueous solution containing the radiolabeled thymine photoproduct that is eluted faster (t_R = 15.3 min) than thymine (t_R = 21.5 min) on the ODS-2 column. The resulting solution was exposed to the UVC radiation (mostly 254 nm) from a germicidal lamp. The photolyzed solution, to which thymine and *cis-syn*-cyclobutadithymine were added, was analyzed by two-dimensional thin-layer chromatography on silica gel plates using eluent systems 1 and 2 (vide supra). The silica gel containing thymine and *cis-syn*-cyclobutadithymine, as detected by fluorescence quenching upon exposure to 254 nm light, was scraped off and counted for radioactivity. A similar approach was used for the control experiment with the exception of the photolysis of the radiolabeled photoproduct, which was omitted.

(*11*) *Spectrometric Measurements.* ^{252}Cf plasma desorption mass spectrometry (PDMS) analysis was performed on an apparatus built in the laboratory (Viari et al., 1989). The photoproducts isolated by HPLC were dissolved in a small volume (10 μL) of a mixture containing propan-2-ol and water and then deposited by electrospray on a thin aluminized Mylar foil using the standard procedure (Mac Neal et al., 1979). The acquisition time was about 1 h. Fast atom bombardment mass spectrometry (FABMS) measurements were made on a Kratos Model MS 50 spectrometer (Manchester, UK) equipped with a FAB gun. Desorption of the molecule was achieved by exposure to a beam of 8 keV xenon atoms of the sample dissolved in a glycerol mull. ^{1}H NMR experiments were carried out on Model WM 250 and AM 400 Bruker spectrometers operating at 250 and 400 MHz, respectively.

(*12*) *Enzymatic Processes and Analysis of Photodamaged Sites in Defined DNA Fragments.* Irradiated DNA samples were digested with 4 units of T4 DNA polymerase 3′–5′ exonuclease in the absence of dNTP. For the enzymatic photoreactivating treatment, about 1 unit/μg of DNA was used in 100 mM NaCl/50 mM Tris-HCl, pH 8.0/1 mM EDTA/1 mM DTT. After a dark preincubation period of 10 min at 37 °C, the mixture was illuminated at 37 °C by 36 kJ m^{-2} of UVA light emitting mostly at 365 nm. For the detection of cyclobutane pyrimidine dimers, DNA was incubated for 20 min at 37 °C with an excess of T4 endonuclease V in 100 mM NaCl/10 mM Tris-HCl, pH 8.0/10 mM EDTA as described in Bourre et al. (1987). In a subsequent step, 1 μg of carrier DNA was added to the enzymic digests and DNA was extracted with chloroform/isoamyl alcohol (19:1 v/v) and precipitated. Digestion products were analyzed on 12% polyacrylamide/urea gels alongside the four Maxam–Gilbert se-

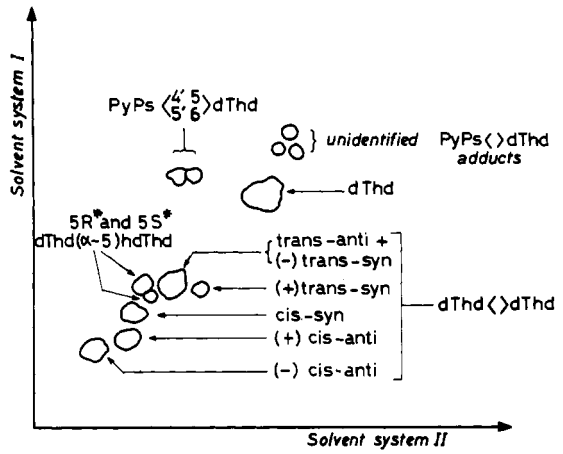

FIGURE 2: Two-dimensional thin-layer chromatography on silica gel plate of the photoproducts resulting from the UVA exposure of a mixture of thymidine and pyrido[3,4-*c*]psoralen (PyPs) in the dry state (solvent systems I and II, see Experimental Procedures).

quencing reactions and visualized as bands on autoradiograms. Slices of gel corresponding to bands were excised and counted for radioactivity. Quantitation of the photolesions was made as described in Sage and Moustacchi (1987).

RESULTS

(1) Identification of the Six Diastereoisomers of Cyclobutadithymidine and of (5R)- and (5S*)-5,6-Dihydro-5-(α-thymidylyl)thymidine.* Exposure of a mixture of thymidine (dThd) and the three pyridopsoralen derivatives PyPs, MePyPs, and 2N-MePyPs in a 10:1 ratio (w/w) as a dry film gave rise, in addition to the expected pyridopsoralen<>thymidine [2 + 2] photocycloadducts, to several nucleoside photoproducts. A typical two-dimensional thin-layer separation of the resulting [$^{14}CH_3$]thymidine photoproducts generated in the presence of PyPs is illustrated in Figure 2. It is noteworthy that the latter photoproducts, which were predominant, exhibit lower chromatographic mobility than the Pso<>dThd photoadducts. Interestingly, these nonfluorescent modified nucleosides, which were shown to consist of eight compounds (vide infra), were generated whatever the pyridopsoralen derivative used as the photosensitizer. The six possible diastereoisomers of cyclobutadithymidine including the cis-syn and trans-syn forms as well as the two pairs of cis-anti and trans-syn diastereoisomers were characterized by the comparison of their ^{1}H NMR and FABMS features with those of the authentic samples (Cadet et al., 1985). Additional relevant information on the structure

Table I: Yield (in Percentage) of the Various Photoproducts That Were Generated by Exposure to 365-nm Light of a Mixture of [$^{14}CH_3$]Thymidine and Three Isomeric Pyridopsoralens in the Dry State

photoproducts	pyridopsoralen derivatives		
	PyPs	MePyPs	2N-MePyPs
(-) *cis-anti*-dThd<>dThd	4.4	3.4	0.3
(+) *cis-anti*-dThd<>dThd	6.8	4.7	0.4
(-) *trans-syn*-dThd<>dThd	3.1	1.6	0.3
(+) *trans-syn*-dThd<>dThd	1.3	1.0	0.2
cis-syn-dThd<>dThd	12.2	9.0	0.8
trans-anti-dThd<>dThd	8.1	5.4	0.4
5*R** and 5*S** spore photoproducts	3.3	1.6	0.3
dThd<>Pso monoadduct I	0.3	*a*	1.5
dThd<>Pso monoadduct II	0.9	*a*	2.8

[a] Not determined.

of the cyclobutadithymidines was provided by an extensive ^{252}Cf-PDMS analysis (Figure 3). The mass spectra exhibited quasi-molecular ions $(M + Na)^+$ and $(M + 2Na - H)^+$ at *m/z* 507 and 529, respectively. These are strongly indicative of a molecular weight of 484, which may be rationalized in terms of a cyclobutane thymidine dimer (dThd<>dThd) structure (Cadet et al., 1986). Further support for the cyclobutadithymidine assignment was provided by the observation of notable fragments at *m/z* 265, 287, and 391 corresponding to $(M - BS + Na)^+$, $(M - BS + 2Na - H)^+$ and $(M - S + H + Na)^+$, respectively, where BS is the thymidine moiety and S is the 2-D-deoxy-*erythro* pentosyl fragment. The observed splitting of the cyclobutane ring, which gives rise to the monomeric thymidine, is characteristic of cyclobutyl-type structures upon electron impact (Fenselau, 1976) as well as soft ionization mass spectrometry analysis (Viari et al., 1989). It should also be noted that these mass spectra (Figue 3b) are similar to those obtained with the dThd<>dThd diastereomers (Figure 3a) prepared by acetone photosensitization (Cadet et al., 1985). The two other photoproducts, which exhibited similar TLC behavior to those of cyclobutadithymidines (Figure 2), were assigned as the 5*R** and the 5*S** diastereomers of 5,6-dihydro-5-(α-thymidylyl)thymidine, the so-called "spore photoproducts" (Varghese, 1970; Cadet & Vigny, 1990). This was achieved by the comparison of their 400-MHz ^{1}H NMR and FABMS features with those of the authentic compounds obtained by far-UV photolysis of thymidine either in the dry state or in frozen aqueous solution (Shaw, 1987).

The yield of formation of both dThd<>dThd and "spore photoproducts" for the three pyridopsoralens investigated was found to decrease in the following order: PyPs > MePyPs >

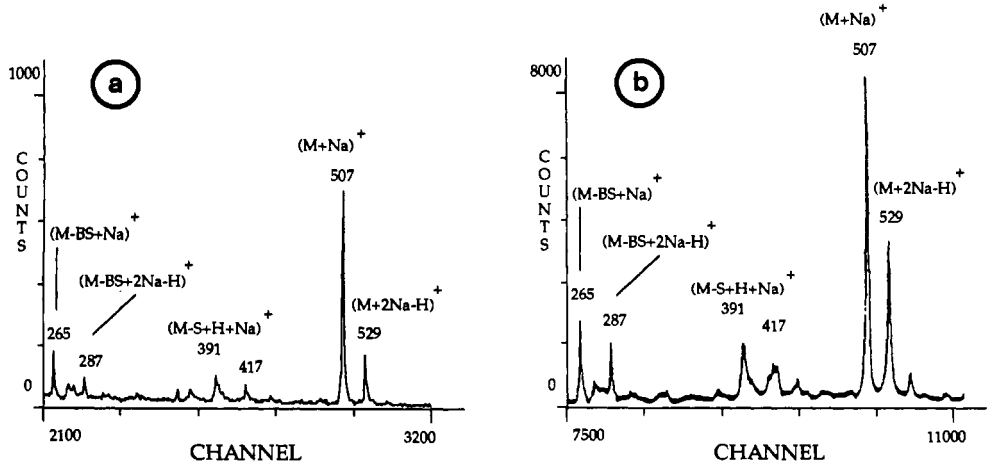

FIGURE 3: ^{252}Cf-PDMS mass spectra in the positive mode of model *cis-syn*-cyclobutadithymidine (a) and of the dimeric thymidine photoproduct generated by exposure of dThd to 365-nm light in the presence of MePyPs (b)

Table II: Photoreversal Experiments of *cis-syn*-Cyclobutadithymine Isolated from DNA Treated by MePyPs and 365-nm Light[a]

	radioactivity in the TLC spot[b]		
	Thy<>Thy	Thy	Thy<>Thy/Thy
before photoreversal at 254 nm	1055	667	1.58
after photoreversal at 254 nm	293	1429	0.21

[a] *cis-syn*-Cyclobutadithymine and thymine were separated by two-dimensional thin-layer chromatography on precoated silica gel plates. [b] Radioactivity in counts per million was determined by scintillation counting of the radioactive material contained in the scraped-off silica gel.

2N-MePyPs (Table I). However, an opposite trend is noted when the rate of near-UV induction of Pso<>dThd photocycloadducts is considered. Another interesting observation dealt with the similarity in the ratio of formation of the two classes of dimeric thymidine photoproducts irrespective of the three pyridopsoralens considered. It should also be mentioned that the formation of cyclobutadithymidines as well as the 5*R** and 5*S** diastereomers of 5,6-dihydro-5-(α-thymidylyl)thymidine was not detected upon exposure of thymidine to UVA radiation in the presence of other psoralens including 3-carbethoxypsoralen, 5-methoxypsoralen, and 8-methoxypsoralen. In addition, 2′-deoxycytidine was not found to undergo detectable photodimerization following pyridopsoralen photosensitization experiments in the dry state.

(2) *Characterization of the cis-syn-Cyclobutadithymine within DNA Exposed to UVA Radiation in the Presence of MePyPs.* The reversed-phase HPLC elution profile of the formic acid hydrolysate of the MePyPs–[^{3}H]thymine DNA complex exposed to 365-nm UVA light, monitored by radioactivity counting, exhibits a main radiolabeled thymine photoproduct (t_R = 15.3 min) besides the overwhelming thymine peak (t_R = 21.5 min). This radiolabeled thymine photoproduct (t_R = 15.3 min) was found to have the same retention time as the coinjected cold *cis-syn*-cyclobutadithymine marker monitored by UV detection at 230 nm (data not shown). In order to confirm the assignment of the Thy<>Thy photoproduct, a photoreversion experiment was carried out on the related radiolabeled compound. A fraction of the HPLC-collected product (t_R = 15.3 min) was cochromatographed with cold *cis-syn*-Thy<>Thy on a silica gel thin-layer plate for two-dimensional analysis (Cadet et al., 1980). The other fraction was exposed to 254-nm radiation in aqueous solution prior to the TLC analysis. From the data (Table II) it is concluded that (1) the radioactive thymine photoproduct had the same TLC properties as the cis-syn isomer of cyclobutadithymine for both eluting systems used in the two-dimensional separation and (2) far-UV irradiation of the photoproduct led to the photoreversion to the parent thymine molecule, as expected for a cyclobutadipyrimidine (Fisher & Johns, 1976; Garcês & Davila, 1982).

The rate of formation of *cis-syn*-cyclobutadithymine within DNA exposed to near-UV radiation in the presence of MePyPs was $1.5/10^3$ thymine bases as determined by radioactivity counting of the two respective compounds following HPLC analysis.

(3) *Measurement of the Two Diastereomeric MePyPs Photocycloadducts to Thymidine within Isolated DNA.* Three radiolabeled hydrophobic thymidine photoproducts were found to be present in the enzymic hydrolysate of the DNA photomodified by MePyPs as inferred from reversed-phase HPLC analysis. It should be noted that under the mild conditions of hydrolysis and chromatographic elution used (H_2O/CH_3OH, 60:40 v/v) the three observed photoproducts, which all fluoresce, are stable (Moysan, 1987; A.M., unpublished data). The two main photoproducts are likely to be the cis-syn diastereomeric pair of [2 + 2] photocycloaddition products of MePyPs to dThd involving the furan side (A.M., unpublished data). The rate of formation of the MePyPs<>dThd photoadducts, determined by radioactivity counting, was $7/10^3$ thymidine residues.

(4) *Detection of Monoadducts and Cyclobutadithymine Photoinduced by MePyPs within DNA Fragments of Defined Sequence.* A combination of three enzymatic probes was used for the detection and the quantitation, at the sequence level, of the two types of DNA photolesions induced by MePyPs, i.e., monoadducts and cyclobutane thymine dimer. The 3′–5′ exonuclease activity associated with the T4 DNA polymerase has been shown to be blocked by bulky adducts, in particular by cyclobutadipyrimidine and pyrimidine(6–4)pyrimidone photoproducts induced by UV at 254 nm (Doetsch et al., 1985) as well as by furocoumarin mono- and biadducts (Sage & Moustacchi, 1987). This enzyme already served as a probe to study the sequence specificity in the photobinding of pyridosporalens and other furocoumarins to DNA (Boyer et al., 1988; Miolo et al., 1989). T4 endonuclease V cleaves DNA specifically at sites of cyclobutadipyrimidines but does not recognize any other known form of base damage (Sancar & Sancar, 1988). DNA photolyase, in conjunction with UVA, reverts cyclobutadipyrimidine to the corresponding pyrimidine monomeric nucleobases and has apparently no effect on other DNA lesions (Sancar & Sancar, 1988).

A 5′-end-labeled DNA fragment, 123 bp long, serving as a substrate for the photoreaction, was enzymatically treated or left untreated. Digestion products were resolved by polyacrylamide gel electrophoresis under denaturing conditions, alongside the Maxam–Gilbert sequencing reaction products (Figure 4a). In such an experiment, DNA was irradiated in the presence of MePyPs at two UVA doses, 54 kJ m^{-2} (lanes 5–8) and 18 kJ m^{-2} (lanes 9–12). As control experiments, DNA was exposed to MePyPs without irradiation (lanes 13–16) or was UVA-irradiated in the absence of MePyPs (lanes 17–20). DNA was also irradiated with UVC light at 254 nm, at a fluence of 2 kJ m^{-2} (lanes 1–4). No significant band pattern appears in lanes 4, 8, 12, 16, or 20 corresponding to undigested DNA or in lanes 13–15 and 17–19 relating to control DNA samples exposed either to the T4 DNA polymerase 3′–5′ exonuclease or to the T4 endonuclease V. In contrast, in lanes 5 and 10 corresponding to DNA photoreacted with MePyPs and digested with the T4 DNA polymerase 3′–5′ exonuclease, bands corresponding to termination products at sites of photolesions appear. From the comparison with the migration of the Maxam–Gilbert sequencing reaction products it appears that termination of digestion occurs at TTTTA, TTA, TTAAT, and TT sites. Furocoumarins, and more specifically pyridopsoralens, have already been shown to preferentially photoreact with such types of sequence (Boyer et al., 1988). When the same DNA samples are digested with the T4 endonuclease V, few of the above bands persist (lanes 6 and 11). These bands correspond to cleavage of DNA at cyclobutadipyrimidine located at TTTTA, TTAAT, and TTA sites. The difference in the migration of the products derived from digestion with the exonuclease or with the endonuclease is due to differences in the terminus of the two kinds of digestion products. The main band appearing at the TTTTA site results in cleavage at a cyclobutadithymine formed between the two 3′ thymine residues. Bands in lanes 6 and 11 are less intense than bands in lanes 5 and 10, indicating that only a fraction of MePyPs-induced photolesions are cyclobutadipyrimidines. The amount of cyclobutadipyrimidines greatly varies from site to site. Figure 4c, which represents a quan-

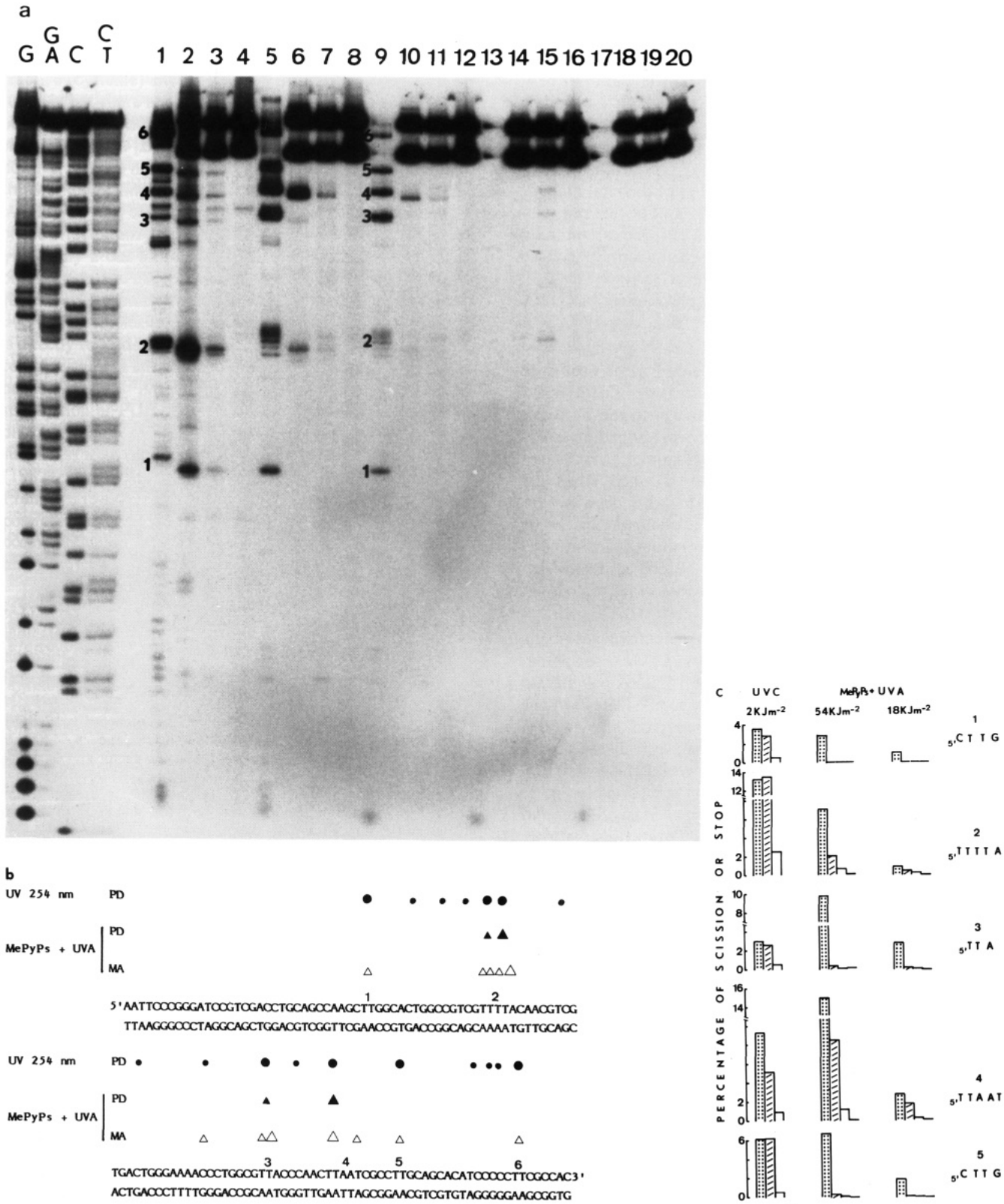

FIGURE 4: Photoinduction of furocoumarin–thymine monoadducts and cyclobutadithymine in defined sequence DNA by MePyPs. (a) Sites of termination or enzymatic cleavage analyzed on 12% polyacrylamide/urea gel. 5′-end labeled DNA was exposed to MePyPs + 54 kJ m^{-2} of UVA light (lanes 5–8), MePyPs + 18 kJ m^{-2} of UVA (lanes 9–12), MePyPs and no UVA (lanes 13–16), or 54 kJ m^{-2} of UVA in the absence of MePyPs (lanes 17–20) or was irradiated at a dose of 2 kJ m^{-2} of UVC at 254 nm (lanes 1–4). In lanes 1, 5, 9, 13, and 17, DNA was digested with T4 DNA polymerase 3′–5′ exonuclease in the absence of dNTP. In lanes 2, 6, 10, 14, and 18, DNA was digested with T4 UV endonuclease. In lanes 3, 7, 11, 15, and 19, DNA was exposed to photolyase plus UVA prior to digestion with T4 UV endonuclease. In lanes 4, 8, 12, 16, and 20, DNA was undigested. (b) Damage distribution corresponding to autoradiogram of part a. Small symbols represent sites of low frequency of photolesions. Larger symbols are "strong" sites for photoproduct formation. Numbers correspond to the numbered bands on the autoradiogram (MA, MePyPs monoadducts; PD, cyclobutadipyrimidines). (c) Quantitation of photolesions at sites showing up on the autoradiogram. Dotted bars represent the termination of T4 DNA polymerase 3′–5′ exonuclease at all photolesions (see text). Dashed bars represent the incision by T4 UV endonuclease at cyclobutane pyrimidine dimers. Empty bars represent the incision by T4 UV enconuclease after photoreactivation of cyclobutadipyrimidine by photolyase plus UVA. The fourth bar in MePyPs-treated lanes corresponds to undigested samples. The level of incision of control DNA samples is of the same order as for the undigested modified-DNA samples.

titation of the photolesions revealed on the autoradiogram of the Figure 4a, shows that at TTTTA site about 30% of the photolesions are cyclobutadipyrimidine, whereas at the TTAAT site it represents more than half of the total photolesions. Cyclobutadipyrimidines at TT in a GC context or at CC are barely detected (Figure 4, part a, lanes 6 and 11, and part c), while they substantially arise at sequences defined as "strong sites" for the photoreaction of psoralen derivatives (Boyer et al., 1988). From quantitation of the total amount of photolesions (from lanes 5 and 10) and of the extent of cyclobutadithymines (from lanes 6 and 11), one can estimate that cyclobutane thymine dimers represent about 20% of the total photolesions induced by MePyPs (0.11 Thy<>Thy/0.59 photolesions or 0.06 Thy<>Thy/0.28 photolesions per DNA molecule after treatment with MePyPs plus 54 or 18 kJ m^{-2}, respectively).

When the MePyPs-photoreacted DNA is subjected to photoreactivation by the combined treatment with DNA photolyase plus UVA, prior to digestion with the T4 endonuclease V, the intensity of the bands corresponding to cyclobutadithymines greatly diminishes (Figure 4, part a, lanes 7 and 12, and part c). Lane 3 of Figure 4a, where DNA was irradiated at 254 nm, photoreactivated, and then digested with the T4 endonuclease V, reveals that the photoreactivation of Thy<>Thy is not complete under our conditions. In any case, these experiments demonstrate that the cyclobutadipyrimidines photoinduced in DNA by MePyPs are photoreactivable by *E. coli* DNA photolyase.

To determine the potential sites of cyclobutane pyrimidine dimers, the same DNA fragment was irradiated with 254-nm UVC light and then digested with the T4 DNA polymerase 3′-5′ exonuclease (lane 1) or T4 endonuclease V (lane 2). Lane 1 reveals the presence of both cyclobutadipyrimidines and 6–4 photoproducts, whereas lane 2 reveals exclusively the sites of cyclobutadipyrimidines. The occurrence of this last UVC-induced lesion along the DNA fragment is reported in Figure 4b, in addition to the sites of formation of monoadducts and Thy<>Thy photoinduced by MePyPs. This reveals that only few potential sites are targets for MePyPs-photoinduced cyclobutadithymine formation. In particular, cyclobutadipyrimidines are not detected at TT sites in a GC environment or at CC sites after treatment of DNA with MePyPs plus UVA, whereas they are present after irradiation of DNA with UVC.

In order to confirm the presence of cyclobutadipyrimidines and to show that these lesions are photoreactivable, whereas monoadducts are not, MePyPs-reacted DNA was subjected to the action of another couple of enzymes (Figure 5). When MePyPs-photoreacted DNA is exposed to the photoreactivating enzyme plus UVA prior to digestion with T4 DNA polymerase 3′–5′ exonuclease, the intensity of critical bands (corresponding to sites of cyclobutadithymines revealed in the previous experiment) diminishes. Because a different amount of radioactivity was loaded in the two lanes, there is a general decrease in intensity of all the bands of the lane where photoreactivation took place. Nevertheless, it clearly appears that the intensity of the two bands corresponding to the TTTTA and TTAAT sites decreases more than the one of the other bands, after photoreactivation. Quantitation of the fraction of radioactivity present in each band confirms this observation. Moreover, it indicates that about 40% of the photolesions at the TTTTA site and 55% of the photolesions at the TTAAT site are cyclobutadithymines, in agreement with the previous experiment. In addition it shows that psoralen monoadducts cannot be photoreversed by photolyase.

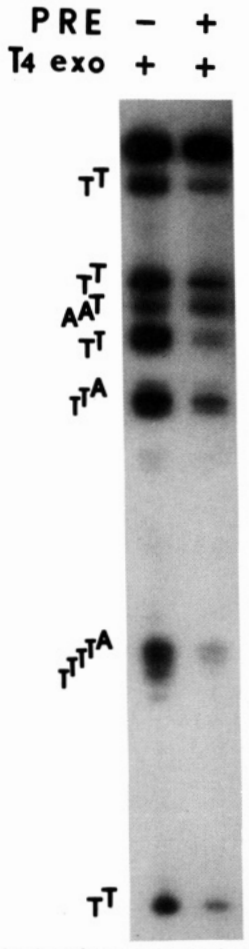

FIGURE 5: Photoreactivation of DNA photolesions induced by MePyPs. 5′-end-labeled DNA was UVA-irradiated at a fluence of 54 kJ m^{-2} in the presence of MePyPs, was exposed or not to photolyase plus UVA (PRE) prior to digestion with T4 DNA polymerase 3′–5′ exonuclease (T4 exo), and was analyzed on 12% polyacrylamide/urea gel. The radioactivity loaded in lane PRE+, T4 exo+ is about 60% of the one loaded in the lane PRE–, T4 exo+.

Discussion

Evidence is provided here that the photoreaction of isomeric pyridopsoralens such as methylpyrido[3,4-*c*]psoralen, 7-methylpyrido[3,4-*c*]psoralen, and 7-methylpyrido[4,3-*c*]psoralen with thymidine in the dry state unexpectedly leads to the formation of dimeric nucleoside photoproducts, in addition to the specific Pso<>dThd [2 + 2] photocycloadducts. The thymidine photoproducts induced by photoexcited pyridopsoralens were characterized as the various possible diastereomers of cyclobutadithymidine and 5,6-dihydro-5-(α-thymidylyl)thymidine. Moreover, *cis-syn*-cyclobutadithymine was found to be generated in a significant yield within DNA treated with MePyPs and UVA light (365 nm).

Cyclobutadipyrimidines and the thymidine "spore photoproducts" are known to be formed as the result of direct effects of exposure of DNA and RNA pyrimidine components to UVB and UVC radiations within the wavelength range 250–320 nm [for reviews see Fisher and Johns (1976), Cadet et al. (1986), and Cadet and Vigny (1990)]. It is interesting to note that exposure of thymidine to 365-nm light, in the absence of the photosensitizing pyridopsoralens, did not result in any detectable formation of the two classes of dimeric thymidine photoproducts. Therefore, it is reasonable to assume that the pyridopsoralen-mediated photoinduction of the diastereoisomers of cyclobutadithymidine and 5,6-dihydro-5-(α-thymidylyl)thymidine is essentially indirect. A reasonable mechanism to explain the formation of dThd<>dThd involves

energy transfer from the pyridopsoralen in a photoexcited long-lived triplet state to a molecule of thymidine in a ground state. In a subsequent step, the resulting triplet excited thymidine is expected to undergo a [2 + 2] dimerization reaction with a second nucleoside. It is worth mentioning that the photophysical properties of the pyridopsoralens inferred from phosphorescence spectroscopic measurements and CNDO/S quantum chemistry calculations are consistent with the mechanism of triplet–triplet energy transfer, which is likely to involve excited vibrational states of the donor (Costalat et al., 1990). This process appears to be specific of pyridopsoralens, as shown by the experiments performed with several lowest triplet energy furocoumarins, including 3-carbethoxypsoralen, 8-methoxypsoralen, and 5-methoxypsoralen. Further support for this energy-transfer mechanism was provided by the lack of detectable cyclobutadipyrimidine formation in the UVA-mediated photoreaction of any of the three pyridopsoralens with 2′-deoxycytidine when irradiated in the solid state. This may be explained in terms of the higher energy triplet level of 2′-deoxycytidine with respect to that of thymidine (Guéron et al., 1967; Meistrich, 1970). The mechanism of formation of the "spore photoproducts" is also likely to be explained in terms of generation of triplet excited thymidine intermediates. It can be noted that the production of pyrimidine(6–4)pyrimidone and/or its Dewar valence isomer, another class of UVC photoproducts, has not been detected.

In model photoreactions involving thymidine, the yield of cyclobutadithymidines induced by photoexcited MePyPs was found to be higher than those of furocoumarin photoaddition products. The situation is different for DNA since the latter photoaddition products are predominant versus cyclobutane thymine dimer (ratio 4.7). The first limiting conditions for the formation of cyclobutadithymine in DNA is the necessity to have two adjacent thymines on the same oligonucleotide strand. Statistically, this represent about 25% of the thymine nucleobases in DNA. Another point to be considered is the mechanism of energy transfer in the formation of cyclobutadithymine. This would require a molecule of MePyPs intercalated in the double helix to be located closely to a potential site of cyclobutane thymine dimer formation. Assuming a triplet–triplet transfer in the generation of Thy<>Thy, the critical distance between the donor (MePyPs) and the acceptor (thymine) should be about 20 Å ($d < 20$ Å), corresponding to about 6 base pairs. The distribution of *cis-syn*-Thy<>Thy in DNA fragments of defined sequence (Figure 4b) is in favor of this mechanism.

The base sequence specificity in the induction of cyclobutane pyrimidine dimers by UVC radiations (mostly 254 nm) has been extensively studied (Gordon & Haseltine, 1982; Brash & Haseltine, 1982; E.S., unpublished data). It can be summarized as follows: TT > TC or CT > CC and a pyrimidine in the 5′ position of a site enhances the cyclobutadipyrimidine formation at this site. The occurrence of cyclobutadipyrimidines induced by UVC and MePyPs plus UVA is given in Figure 4b. Only few potential sites are target for MePyPs photoinduction of these lesions. They are detected neither at TT sites in GC context, which are nevertheless targets for monoadducts formation, nor at CC sites. Cyclobutane pyrimidine dimers are mainly produced at "strong" sites of photoreaction with MePyPs, i.e., AT-rich sites containing adjacent thymines. This implies a stronger interaction of MePyPs with certain types of sequence or a better overlap between psoralen rings and thymine at these sites allowing energy transfer. Considering the extent of DNA modification in our experiments, the formation of a cyclobutadipyrimidine next to a furocoumarin monoadduct on the same molecule is unlikely (for example a TTTTA site) and it is impossible at a TTA site.

The three pyridopsoralens studied in model photoreactions do not give rise to the same level of dThd<>dThd (PyPs > MePyPs ≫ 2N-MePyPs). If the induction of Thy<>Thy in DNA by pyridopsoralens occurs in cells and follows the same order (PyPs > MePyPs ≫ 2N-MePyPs), it could contribute to the photobiological effects of pyridopsoralens. Indeed, MePyPs presents higher lethal and mutagenic effects relatively to the isomeric 2N-MePyPs (Blais et al., 1987). At this point, it can be asked to what extent the Pyr<>Pyr formed after photoreaction of MePyPs contribute to the genotoxicity, assuming that this photoproduct and photoadducts are induced in vivo at the same 1:5 ratio as in vitro. An estimate from data obtained on cell lethality in yeast (Waters & Moustacchi, 1975; Averbeck, 1985; Blais et al., 1987) leads to the conclusion that the amount of Pyr<>Pyr produced by MePyPs is low in comparison to the amount formed at an equitoxic dose of UVC. Indeed the LD37 (lethal dose leaving 37% survivors, i.e., a mean of 1 lethal hit per cell) corresponds to about 750 photoadducts plus 150 Pyr<>Pyr/10^7 nucleotides in the case of MePyPs plus UVA treatment and to about 1250 Pyr<>Pyr/10^7 nucleotides in the case of 254 nm (UVC) exposure. In human cells, the same trend is observed (Nocentini, 1990). On such a quantitative basis, it can be assumed that the contribution of the MePyPs plus UVA photoinduced Pyr<>Pyr is negligible in cytotoxicity.

Moreover, Pyr<>Pyr are induced concomitantly with pyrimidine(6–4)pyrimidone photoproduct in DNA of cells exposed to UVC light (Brash, 1988; Cadet & Vigny, 1990). Their respective contribution in genotoxicity of UVC is still a matter of debate, but it is clear that not all Pyr<>Pyr constitute premutagenic lesions [for review see Brash (1988) and Peak and Peak (1989)]. Consequently, it appears that the relatively high genotoxic effect of pyridopsoralens as opposed to other monofunctional psoralen derivatives cannot be simply explained by the induction of Pyr<>Pyr.

It should be kept in mind however, that the sequence specificity of Pyr<>Pyr induced by MePyPs plus UVA or by UVC differs (Figure 4). Considering the MePyPs photoreaction, we recall that Pyr<>Pyr dimers as well as photoadducts are formed at common sites that are the AT-rich sequences. The proximity of the two types of lesions at specific sites may hamper the repair process and be responsible for the accumulation of single-strand breaks. Closely opposed UVC photolesions has been rendered responsible for mutations in *E. coli* (Schaaper et al., 1987; E.S., unpublished data).

This work extends the spectrum of DNA lesions that can be induced by certain psoralen derivatives. Moreover, any interpretation of the mode of action of pyridopsoralens should take into account the unexpected type of damage described here.

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