Ruusala, Abe Brown, and Marc Gartenberg for valuable disscussions and technical assistance.

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Synthesis and Properties of Novel Psoralen Derivatives

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Received January 11, 1988; Revised Manuscript Received April 19, 1988

ABSTRACT: We have synthesized a set of new trimethylpsoralen derivatives that are characterized by a chain extending from the 4′-position of the furan ring and linked to this ring by an aminomethylene group. The nature of the side chain can be varied widely. In these derivatives, the chains contain either amino or ethylene oxide units for enhanced water solubility and allow the introduction of a thiol or amine group to nucleic acids. These compounds represent the first set of thiolated psoralen derivatives, and their usefulness is demonstrated in several nucleic acid cross-linking experiments. The reagents can be used to create both intraduplex reversible cross-links between the two single-strand partners in a DNA double helix and interduplex reversible cross-links between two DNA double helices.

Psoralens have been used to study nucleic acid structure, damage and repair, and recombination through their involvement in a cross-linking reaction upon irradiation with ultraviolet light (Saffran et al., 1982; Hanson et al., 1976; Wiesehahn et al., 1977). The photochemistry of psoralen has been reviewed extensively (Hearst, 1981; Song & Tapley, 1979; Cantor, 1980). In the dark, psoralens bind to nucleic acids in an intercalative fashion. Upon irradiation with 365-nm light, covalent addition occurs by the formation of cyclobutane adducts between the psoralen and adjacent pyrimidines (Cole, 1970; Kanne et al., 1982). This reaction can lead to the formation of either cross-links between the two strands of a double-stranded molecule or monoadducts to individual strands (Figure 1). Irradiation at 390 nm leads to formation of only monoadducts (Chatterjee & Cantor, 1978). Cross-links can be reversed by irradiation at 254 nm (Rabin & Crothers, 1979).

Psoralen is useful as a structural probe and mutagen partly because its interaction with DNA and RNA is very specific. This allows for modification of nucleic acids in complex nucleoprotein structures, with minimal perturbation caused by reaction with the protein component. Simple psoralen derivatives have been used extensively to study the structure of the ribosome (Wollenzien & Cantor, 1982; Hui & Cantor,

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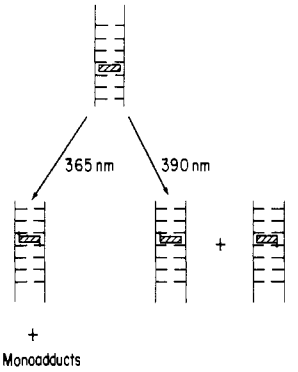

FIGURE 1: Irradiation of DNA at 365 nm in the presence of psoralen results in a mixture of mono- and diadducts. Irradiation of DNA at 390 nm in the presence of psoralen results in only monoadducts.

1985), chromatin (Carlson et al., 1982), and small nuclear RNA (Calvet et al., 1982).

Very few psoralen derivatives are presently available, and these have limited applications. Here we present the synthesis and some preliminary applications of several novel psoralen derivatives. These compounds can be used to introduce reactive sulfhydryls and amines to specific sites in DNA (Saffran et al., 1982) and to form both intra- and interduplex, reversible cross-links. The diverse types of side chains that can be introduced with these methods permit wide variation in the structure and properties of the derivatives. The multifunctionality of these derivatives facilitates many different strategies for controlled placement of the psoralen moiety, thereby extending its range of applications.

Materials and Methods

Synthesis of Psoralen Derivatives. (*A*) *Synthesis of* (*Cystaminylmethyl*)*trimethylpsoralen* (*I*). Cystamine hydrochloride (Aldrich) was recrystallized from hot absolute methanol. A 1.06-g sample of the hydrochloride salt was dissolved in an exact molar amount of 1 N NaOH (9.41 mL). After rotoevaporation of the water, the residue was taken up in 10 mL of hot absolute ethanol. The suspension was centrifuged at 10K rpm for 10 min (4 °C) and the supernatant removed and rotoevaporated, yielding 655 mg of a yellow oil. NMR ($CDCl_3$, Varian T60) δ 3.2–1.7 (8 H, m, $-NCH_2CH_2S$), 1.4 (4 H, s, $-NH_2$).

(Chloromethyl)trimethylpsoralen [28.6 mg (0.10 mmol)] (Isaacs et al., 1977) was reacted with 97.6 mg (0.64 mmol) of cystamine in 2 mL of dry toluene at 65 °C for 2 days. The product was isolated by preparative thick-layer chromatography (silica gel, 1:1 benzene/methanol). The newly formed blue fluorescent band, was scraped off the plate and stirred overnight in 100 mL of benzene/methanol (1:1), and the suspension was centrifuged at 10K rpm for 20 min. The supernatant was rotoevaporated, yielding 18.1 mg of (cystaminylmethyl)trimethylpsoralen (CMT)[1] as a pale yellow oil. NMR (C_6D_6/CD_3OD, 60 MHz) δ 7.8 (1 H, s, phenyl H), 6.2 (1 H, s, lactone H), 3.8 (2 H, s, $-NCH_2Ar$), 3.1–2.7 (8 H, m, $-CH_2CH_2-$), 2.7–2.2 (9 H, m, methyls). Mass spectrum (chemical ionization in methane) 393 (M + 1).

(*B*) *Synthesis of Dithiobis*[*ethylenecarbonyl*(*methylimino*)*ethyleneoxyethyleneoxyethylene*(*methylimino*)*methylene*]*bis*(*trimethylpsoralen*) (*III*). II [8 mg (0.019 mmol)] (Saffran et al., 1982) was dissolved in 50 μL of dry CH_2Cl_2 plus 50 μL of absolute ethanol. Disuccinimidyl dithiobis(propionate) (DTSP, Pierce Chemical Co.) [3.83 mg (0.0095 mmol)] was dissolved in 200 μL of CH_2Cl_2. The DTSP solution was added dropwise into the psoralen mixture over a period of about 1.5 h. The reaction was complete as shown by TLC (silica gel, 1:1 benzene/methanol) with a new spot at R_f = 0.5 that iodine stained, absorbed 254-nm light, and fluoresced under 375-nm light. Also, we noted the disappearance of DTSP and the release of *N*-hydroxysuccinimide. The material was purified by thick-layer chromatography (silica gel, AnalChem, 1 mm, 1:1 benzene/methanol). The band was scraped off and the compound extracted from the gel by stirring overnight in 1:1 benzene/methanol. After centrifuging the suspension, the supernatant was rotoevaporated and the product isolated as a yellow oil (1.8 mg). NMR ($CDCl_3$, 250 MHz) δ 7.8 (2 H, s, phenyl H), 6.25 (2 H, s, lactone H), 3.77 (4 H, $-NCH_2Ar$), 3.8–3.5 [2 OH, m, $-C(O)NCH_2-$, $-CH_2O-$], 3.1–2.7 [18 H, m, $-C(O)NCH_3$, $-SCH_2CH_2C(O)-$, $-CH_2N(CH_3)-$], 2.65–2.4 (24 H, m, methyls excluding amide methyl). Mass spectrum (negative ion in methane) 502 ($^1/_2$M, i.e., split disulfide). Although a cluster centered on 1005 M was evident, instrumental noise in the high molecular weight region was too pronounced to assign it with confidence. The half molecular ion peak, as well as the molecular ion peak, appeared in a lower molecular weight derivative. IR: 1640 cm^{-1} (tertiary amide stretch).

Model reactions involving DTSP and the symmetrical diamine 1,2-bis[2-(methylamino)ethoxy]ethane (II, R = H) led predominantly to the cyclic derivative. This was confirmed by the mass spectrum (CI and EI in methane) and the appearance of the 1640-cm^{-1} IR stretch.

(*C*) *Synthesis of* [[[(*Methylamino*)*ethyl*]*methylamino*]*methyl*]*trimethylpsoralen* (*IVa*). A solution of (chloromethyl)trimethylpsoralen (32 mg, 0.12 mmol) in 5 mL of toluene containing 100 μL ($\sim$1.0 mmol) symmetrical 1,2-dimethyldiaminoethane (Aldrich) was heated at $\sim$130 °C for 24 h under nitrogen. After solvent evaporation, 33 mg of a yellow oil product was isolated as a single spot (TLC) by flash chromatography (silica gel, 14:1 CH_2Cl_2/ammonia-saturated methanol). NMR (300 MHz, $CDCl_3$) δ 7.63 (1 H, s, phenyl H), 6.23 (1 H, broad s, lactone H), 3.58 (2 H, s, $-NCH_2Ar$), 2.8–2.55 (4 H, dt, $-NCH_2CH_2-$), 2.55–2.4 (12 H, m, methyls excluding that on tertiary amine), 2.23 (3 H, s, $-CH_3$ of tertiary amine). Mass spectrum (chemical ionization in methane) 329 (M + 1), 343 (M + 15), 357 (M + 29).

(*D*) *Synthesis of* [[[[[(*Methylamino*)*ethyl*]*methylamino*]*ethyl*]*methylamino*]*methyl*]*trimethylpsoralen* (*IVb*). This was synthesized and completely characterized in a similar manner to the diamine case (IV).

(*E*) *Synthesis of Dithiobis*[*ethylenecarbonyl*(*methylimino*)*ethylene*(*methylimino*)*methylene*]*bis*(*trimethylpsoralen*) (*V*). To a solution of IV (26 mg, 80 μmol) in 2 mL of CH_2Cl_2 was added dropwise a solution of DTSP (14 mg, 35 μmol) in 2 mL of CH_2Cl_2 over a period of 25 min at room temperature. The reaction was complete by TLC (silica gel, 7% ammonia-saturated methanol, 93% CH_2Cl_2). Dithiothreitol in methanol produced a TLC mobility change of the product indicative of the presence of a disulfide group. The product was purified by flash chromatography (Sephadex LH-20, 3%-ammonia saturated methanol/97% CH_2Cl_2). NMR (250 MHz, $CDCl_3$) δ 7.6 (2 H, m, phenyl H), 6.23 (2 H, s, lactone H), 3.6 (4 H, $-NCH_2Ar$), 3.6–3.4 (4 H, dt, $-C(O)NCH_2-$), 2.96–2.7 [14 H, m, $-CN(CH_3)-$, $-C(O)-CH_2CH_2S-$], 2.65–2.4 (18 H, m, ring methyls), 2.28 [6 H,

[1] Abbreviations: CMT, (cystaminylmethyl)trimethylpsoralen; AMT, (aminomethyl)trimethylpsoralen; DBP, dithiobis[ethylenecarbonyl(methylimino)ethylene(methylimino)methylene]bis(trimethylpsoralen); NEM, *N*-ethylmaleimide; DTSP, disuccinimidyl dithiobis(propionate).

s, $ArCH_2N(CH_3)$–]. The resonance at δ 7.6 can appear as a multiplet or a broadened singlet in related compounds. Mass spectrum (CI, ammonia) 831 (M + 1), 863 (M + 33), 417 (split disulfide).

Preparation of Bacteriophage λ DNA and pBR322 DNA. DNA from cesium chloride gradient purified bacteriophage λ cI857 and pBR322 DNA was prepared and purified by standard methods [see Maniatis et al. (1982)].

Cross-Linking Experiments. (A) Intraduplex Cross-Linking with Compound III. Compound III (stock concentration 1 mM in 50% ethanol) was added to bacteriophage λ DNA (cI_{857}; 9 μM in base pairs) in 25 mM Tris, pH 7.5, and 1 mM EDTA (TE) to a final concentration of 3 μM compound III and a final volume of about 250 μL. Irradiation was at 390 nm, at 10 °C. After irradiation, the modified DNA was ethanol precipitated (~5 μg of tRNA carrier) and the pellet was washed with 80% ethanol, dried, and resuspended in TE overnight at 4 °C. Reduction of the resulting cross-links was achieved by incubating in 7 mM DTT at 37 °C for 10 min. Cross-linking and cross-link cleavage were analyzed by electrophoresis.

(B) Interduplex Cross-Linking with Compound V (DBP). DBP (stock concentration 0.23 mM in ethanol) was photoreacted with pBR322 DNA (2.5 μg of DNA) at a DBP/bp ratio of 0, 0.05, 0.1, 0.3, and 0.5, in 0.1 mL of 10 mm Bis-Tris, pH 6.3. The final ethanol concentration in all samples was adjusted to 8%. Irradiation was at 365 nm as described below. Reduction of the resultant cross-links was achieved by incubating at 37 °C in the presence of 0.1 M DTT.

(C) Modification of Intraphage λ DNA with Compound I (CMT). Intraphage λ vir DNA (12.5 μg/250 μL, ~90 μM in base pairs, 10 mM Tris, pH 7.9, 20 mM $MgCl_2$, 6 mM DTT) was irradiated at 365 nm under nitrogen for 5 min at 0 °C in the presence of 20, 40, and 60 μM CMT. After irradiation, aliquots of the modified phage were diluted to 1 mL with buffer, dialyzed against 10 mM HEPES, pH 7.9, and 1 mM EDTA, and reacted with excess [^{14}C]NEM (6 μM). The DNA concentration was determined by absorbance at 260 nm, and the number of CMT photoadducts was determined by liquid scintillation counting using a Beckman LS 3133P liquid scintillation counter and Liquiscint (New England Nuclear) as the liquid scintillation cocktail.

(D) Nonbreakabe Bis-Cross-Link Formation with CMT. Bacteriophage λ was preincubated with 20 μM CMT at a CMT/base pair ratio of 3.75 for 30 min at room temperature in a total volume of 1 mL, under nitrogen. Irradiation was at 365 nm for 5 min as described. Unreacted CMT was removed by dialysis against 10 mM HEPES, pH 7.2, and 1 mM EDTA. Half of the modified phage was reacted with [^{14}C]NEM to quantitate the number of CMT adducts per DNA molecule as described above, and the other half was reacted with 10 μM *p*-phenylenedimaleimide at 30 °C for 1 h. The reactions were stopped by adding DTT to 6 mM at room temperature, followed by phenol extraction, ether extraction, and dialysis. The DNA thus obtained was spread for electron microscopy (Davis et al., 1971), and cross-links were assayed by scoring the number of molecules with small loops.

Irradiation. Irradiation at 365 nm was done with a device similar to that described by Isaacs et al. (1977) which consisted of two 400-W mercury vapor lamps wrapped with aluminum foil, with a 4 cm by a 6 cm window cut into the foil and the windows directed toward the sample holder. The sample holder was a cold finger, with circulating, refrigerated cobaltous nitrate solution [38:2:60 ratio of $Co(NO_3)_2 \cdot 7H_2O$/$NaCl/H_2O$ by weight], at about 5 °C. A fan was used to cool the lamps.

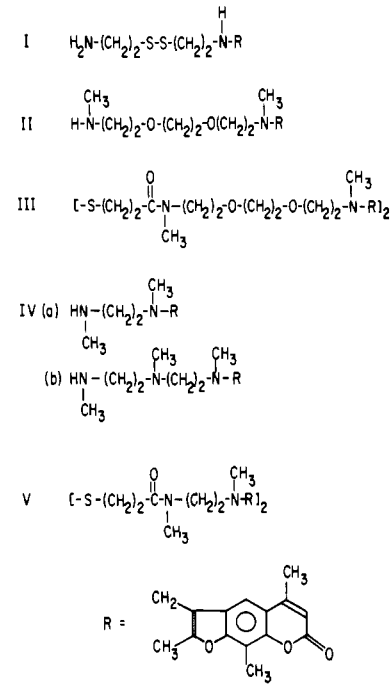

FIGURE 2: Structures of compounds and synthetic intermediates. I, (cystaminylmethyl)trimethylpsoralen; II, [[[[(methylamino)ethoxy]ethoxy]ethyl]methylamino]trimethylpsoralen; III, dithiobis-[ethylenecarbonyl(methylimino)ethyleneoxyethyleneoxyethylene-(methylimino)methylene]bis(trimethylpsoralen); IVa, [[[(methylamino)ethyl]methylamino]methyl]trimethylpsoralen; IVb, [[[[[(methylamino)ethyl]methylamino]ethyl]methylamino]methyl]-trimethylpsoralen; V, dithiobis[ethylenecarbonyl(methylimino)-ethylene(methylimino)methylene]bis(trimethylpsoralen).

Irradiation at 390 nm was done with a Jasco spectral irradiator equipped with interference filters. Irradiations in this instrument were done at 10 °C. Quantitation by photoaddition of [^{3}H]AMT to DNA shows that a molar ratio of AMT/bp = 1 gives approximately 1 monoadduct per 50 base pairs after 2 h of irradiation (data not shown).

Electrophoresis. Agarose gel electrophoresis was carried out in 40 mM Tris, pH 7.5, 20 mM NaOAc, and 1 mM EDTA. The pH was adjusted with acetic acid. The agarose concentrations used are specified in the text. DNA was visualized by ethidium bromide fluorescence. Electrophoresis was done in either a vertical 15 cm long gel or a submerged 20 cm long gel and was generally carried out at about 2.5 V/cm.

RESULTS

Strategy and Design of Psoralen Derivatives. We have synthesized a versatile group of psoralen-based reagents that are highly specific for reaction with nucleic acids after photochemical activation (Figure 2). Since these reagents are to be used in studying biological systems, high water solubility is desirable. This condition was satisfied by choosing water-soluble side chains that contained polar groups or groups that are charged at physiological pH. Several of the new reagents contain a thiol or a disulfide. Thiols can be modified under very mild conditions, and they can be selectively reacted with a number of commonly used reagents. Also, thiols and disulfides can be easily interconverted, opening up the possibility of forming reversible cross-links.

The synthetic route to these derivatives involves only one or two simple steps beyond the synthesis of (chloromethyl)-trimethylpsoralen (Isaacs et al., 1977). In the case of CMT

(Figure 2, compound I), the final compound is produced in a one-step reaction involving (chloromethyl)trimethylpsoralen and an inexpensive, commercially available disulfide-containing chain. The final compound is obtained in a higher yield and by a much simpler procedure than the commonly used (aminomethyl)trimethylpsoralen (AMT). After the photoreaction, CMT can be further reacted by way of the amine or disulfide group.

Hydrophilic oligo- or poly(ethylene oxide) chains are commercially available in a variety of lengths, although most are terminated by hydroxyl groups and a few are terminated with reactive halides (easily synthesized by using, e.g., $SOCl_2$). Straightforward reactions with primary amines convert the halide to a chain terminated by a secondary amine, which is then reacted at a high molar ratio with (chloromethyl)trimethylpsoralen. The resulting psoralen derivative has a tertiary amine near the ring and a secondary amine available for further modification at the other end of the chain (e.g., compound II).

Since polyamines of a variety of lengths are commercially available, the corresponding psoralen derivatives can be made in a one-step reaction. Amines introduced in this way are protonated at physiological pH resulting in increased water solubility and DNA binding. To exemplify a stepwise increase in chain length, compounds IVa and IVb were synthesized.

The psoralen derivatives having secondary amines at the chain end readily react with commercially available succinimide esters, for example, of disulfide-containing compounds, resulting in compounds III and IV. Thus, one can avoid the direct synthesis and protection of sulfhydryl groups. By varying the ratio of chain to (chloromethyl)trimethylpsoralen, one may choose between a monopsoralen and a bis(psoralen) derivative. In the case of a monopsoralen, an extension of the chain in order to synthesize a mercapto derivative is easily accomplished by reaction of the terminal amine with a succinimide ester in either aqueous or nonaqueous solvents.

Cleavable Intraduplex Cross-Linking. The photochemistry of psoralen monoadduct formation has been studied by several groups. Irradiation of calf thymus DNA at 365 nm in the presence of trimethylpsoralen, (hydroxymethyl)trioxsalen, 8-methoxypsoralen, or psoralen leads to the production of both mono- and diadducts. Most of the monoadducts formed in this way result from photoaddition at the 4′,5′ furan double bond. This assignment is based on NMR shifts and splitting patterns, absorption and fluorescence spectra, and pulse–chase irradiation experiments. Psoralen and 8-methoxypsoralen showed an increased yield of pyrone-side monoadducts compared with the other compounds, suggesting easing of steric constraints.

It is also possible to form monoadducts of psoralen to DNA by irradiation at 390 nm (Chatterjee et al., 1978), with no formation of diadducts. This has allowed us to devise a method for introducing cleavable cross-links between the two single-stranded partners of a DNA double helix. The strategy is to add each psoralen moiety of the bis(psoralen) compound III photochemically to opposite strands of a DNA duplex, as shown in Figure 3. Naturally, some of the double additions can be with the same strand. Since the irradiation is at 390 nm, individual psoralen diadducts do not form. Thus, any cross-link that forms should be composed of two psoralen monoadducts bridged by the linker.

Figure 4 shows the results of such an experiment. Bacteriophage λ DNA was photoreacted with compound III as described under Materials and Methods. In lane 1a, the DNA was irradiated in the presence of compound III, treated with

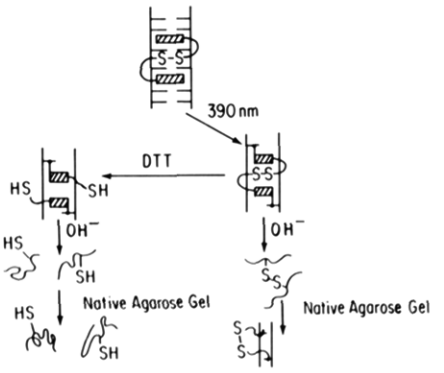

FIGURE 3: Scheme for introducing intraduplex cleavable cross-links with compound III. Psoralen monoadducts are formed by irradiation at 390 nm. Strand separation in the presence of alkali is dependent upon reduction.

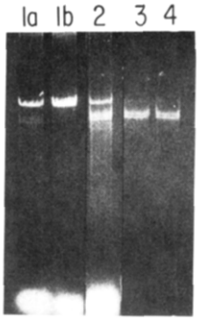

FIGURE 4: Intraduplex cleavable cross-linking of λ DNA with compound III. Lane 1a, DNA irradiated in the presence of compound III, treated with DTT, and denatured with alkali. Lane 1b, same as lane 1a except DTT was omitted. Lane 2, same as lane 1a, but not irradiated. Lanes 3 and 4, irradiated in the absence of compound III and treated with alkali.

DTT, and denatured with alkali (100 mM NaOH at 37 °C for 17 min) prior to electrophoresis. The DNA in lane 1b was treated the same as the DNA in lane 1a except that reduction with DTT was omitted. The DNA in lane 2 was treated the same as the DNA in lane 1a but was not irradiated. In lanes 3 and 4, the DNA was irradiated in the absence of compound III and denatured with alkali prior to electrophoresis. DNA containing a cross-link can be denatured and electrophoresed on native agarose gels, where it renatures rapidly and migrates as a double-stranded molecule. Molecules that do not contain a cross-link will travel as single-stranded molecules. Lanes 3 and 4 show un-cross-linked DNA traveling as two bands, corresponding to the two complementary strands. In lane 2, compound III was added to the DNA solution, but this sample was kept in the dark, so most of the DNA remains in denaturable form. In lane 1b, introduction of cross-links causes the two single-standed partners to renature in the gel, and therefore they travel as double-stranded molecules.

The cross-links formed by irradiation at 390 nm with reagent III should be cleavable by treatment with reagents that can reduce its disulfide bond. Treatment of the cross-linked DNA with 7 mM DTT at 37 °C for 10 min before denaturation with alkali resulted in apparent cleavage of about 75% of the cross-links as shown in lane 1a of Figure 4. The mass of DNA loaded in lanes 1a and 1b was identical: differential staining of single- versus double-stranded DNA probably accounts for the lower staining of lane 1a. It is not clear why some of the cross-links are apparently resistant to cleavage by reduction. This might be explained by the formation of diadducts by stray light, by direct addition to the purine 5-position of thiyl radicals produced through a triplet-state photopathway which could yield cross-links resistant to re-

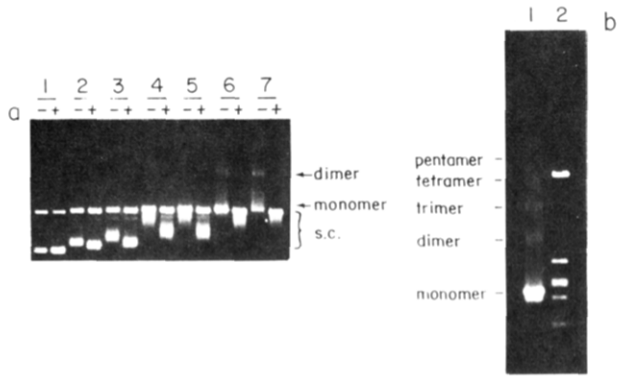

FIGURE 5: Interduplex cross-linking in pBR322 with DBP. The band labeled "s.c." is the supercoiled plasmid.

ducing agents, or incomplete reduction or reoxidation of thiols to the disulfide, but we have no direct evidence pertaining to this problem.

Interduplex Cross-Linking with DBP. DBP (compound V) was designed for studying of nucleic acid conformaton in condensed nucleoprotein structures by forming cross-links between points along a DNA molecule that are in close proximity within the structure. Here we show that cross-links can be introduced between two DNA double helices by irradiation of purified DNA in the presence of DBP.

pBR322 DNA was irradiated in the presence of increasing concentrations of DBP and electrophoresed through a 0.8% agarose gel as shown in Figure 5a, with (+) or without (−) treatment with DTT to break cross-links. In Figure 5a, DNA in lanes 1–7 was reacted at DBP/bp = 0, 0.05, 0.1, 0.18, 0.25, 0.3, and 0.5. Electrophoresis was in 1% agarose in TAE buffer. As the concentration of DBP is increased, higher molecular weight material corresponding to intermolecularly cross-linked material appears above the position of the nicked circular DNA. This higher molecular weight material is abolished by treatment with DTT, consistent with cross-link breakage.

Another interesting feature of DBP cross-linking is its influence on supercoiling. As can be seen in Figure 5a, the photoaddition of DBP to supercoiled DNA results in unwinding, as does the photoaddition of other psoralen derivatives (Wiesehahn & Hearst, 1978). The extent of unwinding due to DBP addition, however, is sensitive to reduction with DTT. We believe this is due to the release of tethered psoralens by disulfide reduction. By tethered psoralen we mean psoralen that is held proximal to the DNA by virtue of the addition of one end of the DBP to the DNA but not the other. The tethered psoralen is free to intercalate into the DNA while still attached through an intact disulfide, thereby influencing DNA topology. Upon reduction, the tethered psoralen is free to diffuse away, resulting in unwinding only by the covalently attached psoralen.

Cross-linked pBR322 molecules can be better resolved on low percentage agarose gels. Figure 5b shows the results of DBP cross-linking in pBR322 electrophoreses in a 0.4% agarose gel. Lane 1 contains pBR322 cross-linked at an input DBP/bp ratio of 0.5 (2.5 μg of DNA/100 μL). Irradiation was at 365 nm for 5 min as described above. Multimers of pBR322 up to a 5-mer can be seen. Lane 2 contains a λ–*Eco*R1 digest molecular weight marker. Electrophoresis was in 0.4% agarose at 2.5 V/cm.

Quantitation of Thiol/Disulfide-Containing Psoralen Derivatives Photoreacted with DNA. There are a number of ways to quantitate the number of psoralens photoreacted with DNA. Radioactive derivatives allow for the most direct determination. Psoralens that form intraduplex crosslinks can

Table I: Quantitation of CMT Adduction to Intraphage λ DNA

CMT/DNA (bp)	cpm/μg of DNA	CMT/DNA (mol/mol)
0.00	36.4	0
0.27	80.9	78
0.53	104.6	125
1.07	138.0	187

Table II: Cross-Link Formation by CMT–*p*-Phenylenedimaleimide

	no. of molecules screened			no. of molecules screened	
no. of small loops	cross-linked sample	control sample	no. of small loops	cross-linked sample	control sample
0	26	54	3	17	8
1	44	31	4–7	12	8
2	23	21			

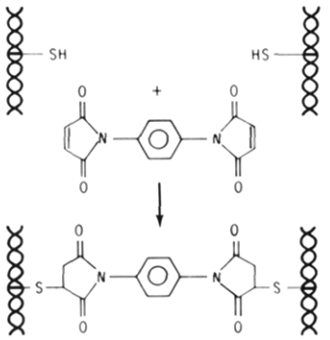

FIGURE 6: Interduplex cross-linking scheme using CMT and *p*-phenylenedimaleimide.

be determined by either their effect on the mobility of restriction fragments in denaturing gels (Cech, 1981), their effect on the kinetics of renaturation in native gels, electron microscopy, or antibody precipitation. The psoralen derivatives that contain a thiol or disulfide can be quantitated by reaction with radioactive NEM.

Table I shows the results of an experiment where DNA was modified with various amounts of CMT and then reacted with radioactive NEM. As the input CMT/DNA ratio increases, increasing levels of modification are observed. This method allows one the convenience of using radioactivity to quantitate the number of psoralens without the inconvenience of having to synthesize radioactive forms of the reagent and should be applicable to all of the thiol-containing psoralen derivatives.

Interduplex Cross-Linking with CMT. The new psoralen derivatives described in this paper place chemically reactive groups at the positions of the DNA where they become attached. The reaction between DNA and CMT leaves a reactive amine and a protected thiol, with which other reactions can subsequently be performed, such as reactions typically used to probe the structures of protein complexes. Here we show that CMT can be used in conjunction with the bifunctional reagent *p*-phenylenedimaleimide to introduce interduplex cross-links between parts of a DNA molecule that are held proximal in a nucleoprotein complex. First, intraphage DNA is modified with CMT in the presence of DTT as described under Materials and Methods. Cross-links are formed by subsequent reaction of these thiols with *p*-phenylenedimaleimide. The number of CMT adducts per DNA molecule was about 100, as determined by radioactive NEM labeling. The resulting tethered thiols were then cross-linked by reaction with *p*-phenylenedimaleimide (Figure 6). Cross-links were deduced by examining small loops in the molecules by electron microscopy. Large loops occurred frequently in the un-cross-linked controls and are therefore not considered. The results of this experiment are shown in Table II. Only loops smaller

than ~2000 bp were scored. A larger number of loops appeared in the CMT-treated sample than in the untreated sample.

Discussion

Simple psoralens have been used extensively to study nucleoprotein structures and repair processes. The derivatives we have synthesized extend the range of problems that can be addressed with psoralen photochemistry. These synthetic procedures were designed to allow the synthesis of related compounds simply by altering the nature of the side chain. Thus, by using different combinations of available diamines and amine-reactive compounds, psoralen derivatives can be tailored to suit a wide range of experimental problems.

The new reagents presented here allow sulfhydryl groups to be introduced to a specific nucleic acid site and are capable of both inter- and intraduplex, reversible cross-linking. These derivatives, as well as their synthetic precursors, contain an aminoalkyl group to enhance their water solubility and reactivity with DNA. The variety of water-soluble chains that can be used is virtually unlimited, ranging from naturally occurring polypeptides and sugars to synthetic polyamides, acrylic acids, and poly(alkylene oxides). Since thiols are highly reactive, their introduction onto DNA allows for quantitation by reaction with radioactive electrophiles. Attachment of psoralen to proteins and attachment of spectroscopic probes to nucleic acids through the thiol, disulfide, or amine groups of these derivatives may find useful application. Also, mercurated supports might be useful in conjunction with these compounds.

There are a few limitations in the use of these reagents. One constraint is that it unwinds the duplex, possibly leading to significant structural perturbations. In addition, some studies suggest that psoralen modification may lead to DNA kinking [Peckler et al., 1982; but see, however, Sinder and Hagerman (1984)]. Low levels of cross-linking should minimize these problems. Also, a disulfide bond may not be an ideal cleavable linker for use in vivo since reducing agents in the cell (e.g., glutathione) are capable of breaking this linkage.

We have used DBP and related compounds to study the structure of bacteriophage λ and the animal virus SV40 (Schwartz et al., 1983; Haas et al., 1983; Welsh & Cantor, 1987).

Registry No. I, 115305-54-9; II, 83353-03-1; III, 115289-65-1; IVa, 115289-66-2; IVb, 115289-67-3; V, 112250-61-0; cystamine, 51-85-4; (chloromethyl)trimethylpsoralen, 62442-57-3; disuccinimidyl dithiobis(propionate), 57757-57-0; 1,2-dimethyldiaminoethane, 110-70-3.

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