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Nucleotide Positions Responsible for the Processivity of the Reaction of Exonuclease I with Oligodeoxyribonucleotides†

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ABSTRACT: The processive hydrolysis of single-stranded oligodeoxyribonucleotides by exonuclease I from *Escherichia coli* has been investigated. Oligodeoxyribonucleotides and their analogues, which contain either an abasic site or a methylphosphonate internucleotide linkage, were partially hydrolyzed by exonuclease I. The relative dissociation constant for the enzyme and each oligomeric product was calculated from the concentration of that oligomer found in solution and hence released by the enzyme before complete hydrolysis. The results have led to a characterization of the two oligodeoxyribonucleotide domains that bind to exonuclease I. The first domain, which begins at the reactive 3′-terminal phosphodiester and extends to the 7th nucleoside base, requires both phosphodiester monoanions and base residues for its interaction with the enzyme. The second domain includes phosphodiester monoanions in positions 9–13 from the 3′-terminus but does not require nucleoside bases. Methylphosphonate substitutions indicate that only two or three of these phosphodiesters, in variable positions, must remain anionic in order to obtain full enzyme binding. The residues between the two binding domains do not play a significant role in the enzyme–oligomer interaction.

Exonuclease I from *Escherichia coli* catalyzes the hydrolysis of mononucleotide 5′-phosphates from the 3′-terminus of single-stranded DNA (Lehman, 1960; Lehman & Nussbaum, 1964) in a highly processive reaction (Thomas & Olivera, 1978; Brody et al., 1986). Experiments with phosphorothioate analogues have shown that the stereochemical course of the reaction is inversion of configuration at phosphorus and that phosphodiester bond cleavage is at least partially rate limiting (Brody & Doherty, 1985). Kinetic investigations indicate that the maximum rate of hydrolysis is independent of polymer size, and competition experiments have shown that the enzyme associates directly with the 3′-terminus of a polydeoxyribonucleotide (Brody et al., 1986).

An important feature of many enzymes that react with nucleic acids is their ability to remain bound to the polymeric substrate between successive catalytic events (Kornberg, 1980). Exonucleases that react by such a processive mechanism have

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been isolated from many sources including bacteria and bacteriophage (Nossal & Singer, 1968; Thomas & Olivera, 1978; Das & Fujimura, 1980; Joseph & Kolodner, 1983; Joannes et al., 1985), fungus (Chow & Fraser, 1983; Chow & Resnick, 1987; Burgers et al., 1988), and mammalian cells (Lasater & Eichler, 1984; Becerra & Wilson, 1984). In no case, however, is the mechanism by which an enzyme achieves processivity understood.

A previous study from this laboratory has shown that exonuclease I remains associated with a polydeoxyribonucleotide homopolymer during successive nucleotide hydrolyses until the polymer has been degraded to a decamer (Brody et al., 1986). Dissociation then begins to compete with further hydrolysis, and a distribution of decameric and smaller products is obtained. The quantity of each oligomeric product recovered after limited hydrolysis was used to calculate the relative affinity of the enzyme for that oligomer. These results led to a qualitative model for exonuclease I binding in which two nucleic acid binding sites were postulated: an anchor site that includes the 10th and 11th nucleotides from the 3'-terminus and an extended catalytic site that includes the reactive 3'-terminal phosphodiester and several adjacent nucleotides (Brody et al., 1986).

In this paper the two binding domains have been further characterized in terms of the nucleotide positions that interact with exonuclease I. In addition, the contribution of individual phosphate groups and nucleoside bases to binding has been investigated. The effect of the anionic phosphates has been studied via the synthesis of oligomers that contain uncharged methylphosphonate linkages in specific positions. Similarly, the effect of nucleoside bases has been studied with oligomers that contain abasic nucleosides in defined positions. DNA that contains methylphosphonate linkages (Miller et al., 1982; Nobel et al., 1984) and DNA that contains abasic sites (Brunelle & Schleif, 1987, 1989; Feavers et al., 1989) have been used, in other systems, as probes of the contribution of phosphate monoanions and nucleoside bases to protein–nucleic acid binding.

Experimental Procedures

Materials. $(dA)_{16}$, $p(dA)_{16}$, $(dT)_{16}$, $p(dT)_{16}$, polynucleotide kinase, and calf intestine phosphatase were purchased from Pharmacia-LKB. Triethylamine (gold seal), ethylenediamine, and piperidine were obtained from Aldrich, acetic acid (aldehyde free) was obtained from Baker, acetonitrile was obtained from Burdick and Jackson, ammonium sulfate was obtained from Sigma (Molecular Biology Reagent), and (γ-^{32}P)ATP was obtained from New England Nuclear.

General Methods. All dialysis of oligodeoxyribonucleotides was done at 4 °C in 1000 MW cutoff tubing (Spectrum). The oligodeoxyribonucleotide concentration was determined by UV absorbance (Cassani & Bollum, 1969). The absorbance of the oligothymidylates was measured at 265 nm, and an extinction coefficient of 8.6 mM^{-1} cm^{-1} per nucleotide was used. The absorbance of oligodeoxyadenylates was measured at 260 nm, and an extinction coefficient of 10 mM^{-1} cm^{-1} per nucleotide was used. Concentrations are reported in terms of polymer chains rather than total nucleotide content.

HPLC. All HPLC work was done a Beckman Model 332 gradient liquid chromatograph with an LCD variable-wavelength detector and a Hewlett-Packard 3396A integrator. Reverse-phase chromatography was done with Waters μBondapak C18 analytical columns (0.39 cm × 30 cm) and semipreparative columns (0.78 cm × 30 cm). The analytical columns were eluted at 1 mL/min and the semipreparative column was eluted at 2.5 mL/min with linear gradients that were prepared from mixtures of buffer A (0.1 M triethylammonium acetate, pH 7.0) and buffer B (acetonitrile). All columns were run at room temperature and monitored at 265 nm. The different gradients are described in the synthesis procedures for each oligomer. Fractions that contained product were dried by rotary evaporation, redissolved in water, and dried a second time.

Anion-exchange chromatography was done with a Synchrom AX100 column (0.41 cm × 10 cm) that was eluted at 1 mL/min with a linear gradient composed of buffers C (0.05 M potassium dihydrogen phosphate, pH 5.4, 20% acetonitrile) and D (0.05 M potassium dihydrogen phosphate, pH 5.4, 1 M ammonium sulfate, 20% acetonitrile). The gradient went from 0% D to 50% D over 50 min. Thymidylate oligomers were monitored at 265 nm, and deoxyadenylate oligomers were monitored at 260 nm.

Oligodeoxyribonucleotide Synthesis. Oligodeoxyribonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer with β-cyanoethyl phosphoramidite chemistry (Caruthers, 1985) and commercial reagents (Applied Biosystems). Oligomers with only normal oligonucleotide linkages (e.g., 5'-GATCTGGAATTCAAGCTT-3') were synthesized by the manufacturer's recommended protocols. Oligomers used as exonuclease I substrates were synthesized on 1-μmol columns, and oligomers used as size standards were synthesized on 0.2-μmol columns.

Methylphosphonate-Substituted Oligodeoxyribonucleotides. Methylphosphonate linkages were introduced at specific positions in the course of oligothymidylate synthesis (Bower et al., 1987a) via the use of methylphosphonamidite monomers (Applied Biosystems). The oligomers were released from the column with a 1:1 solution of dry ethanol and ethylenediamine by a modification of the procedure of Miller et al. (1986) as described in the Applied Biosystems product literature.[1]

$MP^{13}(dT)_{16}$,[2] synthesized as described above, was redissolved in 1 mL of buffer A and purified by reverse-phase HPLC on the semipreparative column with a gradient that went from 10% B to 13% B in 1 min and then changed again from 13% B to 14.5% B over 75 min. Diastereomer A peak eluted at ~71 min, and diastereomer B peak eluted at ~76 min. The yield of purified products was 0.3 μmol (30%), which was equally distributed between the two diastereomers.

The position of the methylphosphonate linkage in the 16-mer was confirmed by base degradation with aqueous piperidine (Miller et al., 1983). The degradation products were labeled in the 5'-position with ^{32}P (Maniatis et al., 1982) and analyzed on a 20% polyacrylamide gel (Brody et al., 1986). The gel contained a band for the starting material and a band for $p(dT)_{13}$, the expected product of degradation and 5'-phosphorylation (Murakami et al., 1985).

$MP^{6}(dT)_{16}$ was synthesized and chromatographed as described for $MP^{13}(dT)_{16}$. Diastereomer A eluted at ~71 min, and diastereomer B eluted at ~75 min. The diastereomers were dried by rotary evaporation, redissolved in 1 mL of 0.07 M Tris·HCl buffer (pH 8.0), dialyzed at 4 °C against

[1] Applied Biosystems User Bulletin, DNA Synthesizer Model 380, Issue No. 43, Oct 1, 1987.

[2] $MP^{13}(dT)_{16}$ refers to an oligodeoxyribonucleotide with 16 thymidine residues that contains a single methylphosphonate internucleotide linkage in the 13th phosphodiester position from the 3'-terminus. $AP^{14}(dT)_{16}$ refers to an oligodeoxyribonucleotide 16-mer that contains an abasic site (also called an apurinic/apyrimidinic site) located in the 14th base position from the 3'-terminus. $B_{10}P_9$ represents the 10th nucleoside base position (B) and the 9th phosphodiester position (P) from the 3'-terminus. Other methylphosphonates, abasic sites, and nucleotide positions are represented in analogous fashion.

deionized water, and frozen until use. The combined yield of purified products was 0.14 μmol (14%), which was equally distributed between the two diastereomers.

The structure of the product was confirmed by degradation studies as described for $MP^{13}(dT)_{16}$. The gel contained bands for the starting material and the phosphorylated degradation products $p(dT)_{10}$ and $p(dT)_6$. The gel also contained a band that migrated slightly farther than $p(dT)_{10}$. This band has been tentatively identified as a derivative of $p(dT)_{10}$ that contains a methylphosphonate on its 3′-hydroxyl group (Murakami et al., 1985).

$MP^{13,14}(dT)_{16}$ was synthesized by the procedures described above, and the four diastereomers were purified with a gradient that went from 12% B to 16% B over 80 min. Diastereomers A, B, C, and D eluted at ~61, ~65, ~75, and ~79 min, respectively, with relative peak areas of (1):(1.2):(1.9):(1.8). The peak for diastereomer C was broad and in some chromatograms could be resolved into two peaks. The samples were prepared for assay with the procedure described for $MP^6(dT)_{16}$, and the total yield of purified product was 0.15 μmol (15%).

The structural study of $MP^{13,14}(dT)_{16}$ was done by the procedure described for $MP^{13}(dT)_{16}$ except that the products were analyzed by ion-exchange HPLC. A partial degradation of $MP^{13,14}(dT)_{16}$ would be expected to yield starting material, $(dT)_{13}$, and three degradation products that contain methylphosphonates and methylphosphonate fragments (Murakami et al., 1985). Peaks for the starting material and dT_{13} were clearly identifiable after degradation of each of the four diastereomers. An additional peak of similar size was also detected. It has not been determined whether the remaining two degradation products elute at positions that overlap with the three detected peaks or whether these products were produced in low concentrations.

$AP^{14}(dT)_{16}$. An oligodeoxyribonucleotide 16-mer was synthesized with 15 thymidine nucleosides and a single deoxyadenosine nucleoside in the 14th position from the 3′-terminus. The sample of $(dA)^{14}(dT)_{16}$ was purified on the analytical reverse-phase column with a gradient that went from 13% B to 14.5% over 60 min. The product eluted at ~20 min with a yield of 0.8 μmol (80%).

The adenine base was removed from the oligodeoxyribonucleotide to form an abasic site by incubation at pH 1.6 for 24 h at 37 °C (Turler, 1971). The reaction was neutralized, and the resulting $AP^{14}(dT)_{16}$ was purified under the same HPLC conditions used for $(dA)^{14}(dT)_{16}$. The product, which eluted at ~15 min, was dialyzed against water, lyophilized, and redissolved in 1 mL of water. Dialysis was necessary because lyophilization of the abasic product in the HPLC buffer resulted in extensive degradation (Vasseur et al., 1986). The combined yield for the depurination reaction and purification of the abasic oligomer was 0.4 μmol (50%).

The structure of $AP^{14}(dT)_{16}$ was confirmed by base degradation in 0.2 M sodium hydroxide at 100 °C for 35 min (Turler, 1971). The hydrolysis product was dephosphorylated with phosphatase (Maniatis et al., 1982) and analyzed by ion-exchange HPLC. The chromatographic profile clearly showed that the abasic oligomer was converted to $(dT)_{13}$, as expected.

$AP^7(dT)_{16}$. $(dA)^7(dT)_{16}$ was synthesized, purified, and converted to $AP^7(dT)_{16}$ by a procedure similar to that used for $(dA)^{14}(dT)_{16}$. The structure of $AP^7(dT)_{16}$ was determined as described for $AP^{14}(dT)_{16}$, and the main products were, as expected, $(dT)_9$ and $(dT)_6$.

Size Standards. In order to confirm the identities of the degradation products, representative methylphosphonate and abasic products were independently synthesized and chromatographed without purification. The identities of all the products could clearly be determined from the size standards and the chromatographic profiles.

Exonuclease I Degradations. Exonuclease I was purified from an overproducing strain of *E. coli* K12 (Prasher et al., 1983) as described by Brody and Doherty (1985) and stored in a 50% glycerol solution at −20 °C. Each enzyme preparation that was used in this study was reacted with a standard solution of $(dT)_{16}$. The product distributions for all the preparations were indistinguishable, regardless of whether or not the enzyme had lost some of its original activity.

Oligodeoxyribonucleotides and their analogues were reacted with exonuclease I under conditions that were slightly modified from those described previously (Brody et al., 1986). A typical reaction mixture contained the following in 0.12 mL: 67 mM Tris·HCl (pH 8.5), 6.7 mM magnesium chloride, 20 mM 2-mercaptoethanol, 0.1% bovine serum albumin, ~0.03 mM oligodeoxyribonucleotide, and ~0.2 unit of exonuclease I. The reactions were incubated for 20 min in a 1.5-mL plastic Eppendorf tube, quenched by rapid freezing in a dry ice–2-propanol bath, and stored frozen until HPLC analysis. The amount of exonuclease I actually used for each oligodeoxyribonucleotide was varied in preliminary reactions to achieve between 10% and 40% hydrolysis, conditions where the product distributions are independent of the extents of reaction (Brody et al., 1986).

The product distributions for all the dA and dT oligomers were determined by ion-exchange HPLC. In most cases, the column separated 3-mers through 14-mers with base-line resolution. The 15-mers overlapped the large peak due to unhydrolyzed starting materials and could not be quantified. Reactions without enzyme were run for each oligomer and analyzed by HPLC in order to control for the contribution of impurities to the chromatograms of the reaction mixtures. The peak area for each product was corrected for the number of UV-absorbing nucleotides in that oligomer (Brody et al., 1986).

The oligodeoxyribonucleotide 5′-GATCTGGAATTCAAGCTT-3′ was labeled in the 5′-position with ^{32}P and reacted with exonuclease I, and the products were analyzed by gel electrophoresis (Brody et al., 1986). Previous work has shown that gel electrophoresis gives product distributions similar to those obtained via ion-exchange HPLC for the hydrolysis of phosphorylated oligomers (Brody et al., 1986).

Results

Product Distributions

Oligodeoxyadenylate and Oligothymidylates. The product distributions for the reactions of $(dT)_{16}$, $p(dT)_{16}$, $(dA)_{16}$, and $p(dA)_{16}$ with the enzyme (Table I) all contain a small percentage of molecules that are 11-mers or larger and a distribution of smaller products that is maximal at the 9-mer. These results are similar to those found previously for the hydrolyses of oligodeoxyadenylate and oligothymidylate 12-mers (Brody et al., 1986). Although no 11-mer was detected as a product in the previous study, this may have been due to an inability to resolve the small quantities of 11-mer from unhydrolyzed starting 12-mer. The product distributions for $p(dT)_{16}$ and $p(dA)_{16}$ contain a lower percentage of nonamer than was found in the hydrolyses of $p(dT)_{12}$ and $p(dA)_{12}$. The higher amount of nonamer released into solution in the 12-mer study may have been due to a partial inactivation of the enzyme or perhaps to an undetected difference in reaction con-

Table I: Product Distributions

Exo I substrate	% of total products[a]													
	15-mer	14-mer	13-mer	12-mer	11-mer	10-mer	9-mer	8-mer	7-mer	6-mer	5-mer	4-mer	3-mer	2-mer
$(dT)_{16}$	*b*	1.5	1.0	1.4	2.4	7.6	29.1	20.4	12.7	12.5	8.7	2.7	ND	
$p(dT)_{16}$	*b*	*b*	0.9	1.0	1.0	3.4	30.4	29.1	14.5	13.0	5.1	1.6	ND	
$d(A)_1^6$	*b*	*c*	0.5	0.7	4.0	9.6	20.1	8.8	7.1	13.8	19.1	16.3	ND	
$p(dA)_{16}$	*b*	*c*	0.3	0.5	0.9	3.6	24.4	12.3	8.2	14.3	16.1	13.2	6.2	ND
$MP^{13}(dT)_{16}$ (diastereomer A = B)[d]	*b*	1.1	1.3	1.4	2.0	8.6	34.0	33.0	13.2	5.4	ND			
$MP^{6}(dT)_{16}$ (diastereomer A)	*b*	13.8	45.7	32.6	7.9	*e*								
$Mp^{6}(dT)_{16}$ (diastereomer B)	*b*	3.5	5.7	11.3	50.9	13.0	8.0	3.8	3.8	*c*				
$MP^{13,14}(dT)_{16}$ (diastereomer A = B)[d]	*b*	2.7	7.1	26.1	31.2	12.5	10.1	10.3	ND					
$MP^{13,14}(dT)_{16}$ (diastereomer C = D)[d]	*b*	1.4	1.5	4.0	8.2	19.3	29.9	23.9	11.8	ND				
$AP^{14}(dT)_{16}$	*b*	1.2	0.8	0.9	2.4	13.9	51.1	21.5	6.1	2.1	ND			
$AP^{7}(dT)_{16}$	*b*	2.5	1.4	4.1	37.7	54.3	ND[f]							

[a] The product distributions were determined from the averages of between 3 and 5 individual reactions and HPLC analyses. The average of the relative standard deviations for the 92 entries in the table is 14%. ND means that no peak was detected. [b] The peak was not resolved from the large peak due to unhydrolyzed starting 16-mer. [c] Peak obscured by impurities in the starting oligomer. [d] The two diastereomers gave indistinguishable product distributions. [e] No degradation past the 11-mer occurred, even with 100 times more enzyme. [f] $AP^7(dT)_{16}$ could be hydrolyzed completely to small molecules (<4-mer) by treatment with 100 times more enzyme.

ditions.[3] As described under Experimental Procedures, each enzyme preparation used in the current study was reacted with a standard solution of $(dT)_{16}$ to ensure that the reaction resulted in the same product distribution.

Analogues with Methylphosphonate Linkages. $MP^{13}(dT)_{16}$ has a single methylphosphonate linkage in the 13th phosphodiester position from the 3′-terminus. The two diastereomers of $MP^{13}(dT)_{16}$, which occur because the chemical synthesis of the chiral methylphosphonate linkage is not stereospecific, give indistinguishable product distributions when reacted with exonuclease I (Table I).

$MP^{6}(dT)_{16}$, which contains a single methylphosphonate linkage in the 6th phosphodiester position from the 3′-terminus, has two diastereomers with very different product distributions (Table I). None of the products of diastereomer A are smaller than $MP^1(dT)_{11}$, even in the presence of a 100-fold higher concentration of exonuclease I. In contrast, the hydrolysis of diasteromer B proceeds past $MP^1(dT)_{11}$ to $(dT)_{10}$. The slow hydrolysis of $MP^1(dT)_{11}$ results in a greater release of $(dT)_{10}$ into solution than occurs upon hydrolysis of $(dT)_{11}$ (Table I). Snake venom phosphodiesterase has also been found to hydrolyze methylphosphonate internucleotide linkages but in a reaction that is not stereospecific (Noble et al., 1984).

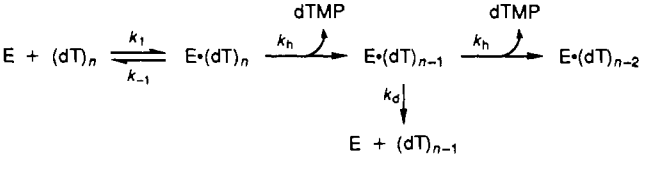
$MP^{13,14}(dT)_{16}$ has two methylphosphonates in phosphodiester positions 13 and 14 from the 3′-terminus. The four diastereomers of this molecule have been separated and individually reacted with exonuclease I. Diastereomer A has the same product distribution as diastereomer B, and diastereomer C has the same product distribution as diastereomer D (Table I). The equivalence of the results with diastereomers C and D indicates that the unidentified impurity in diastereomer C, which was detected by HPLC analysis, does not affect the product distribution. Hydrolysis of diastereomers A or B results in a higher percentage of 11-mers and larger products than does hydrolysis of diastereomers C or D.

Analogues with Abasic Positions. $AP^{14}(dT)_{16}$ has an abasic site in the 14th nucleoside from the 3′-terminus. The abasic position is relatively stable at neutral pH and is expected to be ~99% in the cyclic hemiacetal form (Wilde et al., 1989). The distribution of products larger than a decamer is very similar for this molecule and for $(dT)_{16}$ (Table I). $AP^7(dT)_{16}$ contains an abasic position in the 7th nucleoside from the 3′-terminus. This molecule, under normal reaction conditions, yields products from $AP^5(dT)_{14}$ through $AP^1(dT)_{10}$ (Table I). A large excess of enzyme hydrolyzes $AP^7(dT)_{16}$ completely to small molecules. It is likely that the hydrolysis of $AP^1(dT)_{10}$ occurs at a very slow rate and that the $(dT)_9$ produced is rapidly hydrolyzed to smaller molecules by the large amount of enzyme. While the phosphodiester linkage between an oligodeoxyribonucleotide chain and a 3′-terminal abasic site is resistant to snake venom phosphodiesterase (Stuart & Chambers, 1987), it can be cleaved slowly by other nucleases (Bailly & Verly, 1987; Weinfield et al., 1989).

"Native" Oligodeoxyribonucleotide. The only oligodeoxyribonucleotide in this study that contains all four bases is 5′-pGATCTGGAATTCAAGCTT-3′. The products produced by hydrolysis of this 18-mer, which was designed as one strand of a DNA linker that contains restriction enzyme cleavage sites, were analyzed by gel electrophoresis. The product distribution, expressed as percentages of the total resolved products, was 10-mer (2%), 9-mer (40%), 8-mer (29%), and 7-mer (29%). Products smaller than a 7-mer were produced, but they were not quantitated due to interference with gel impurities. Although quantitation of the smaller oligomers would change the percentage of each product, it is clear that hydrolysis of this "native" sequence of DNA gives results similar to those obtained via hydrolysis of homopolymers (Brody et al., 1986). This reaction, like the reactions previously reported, was not run on a larger scale in an attempt to determine whether any oligomers greater than the 10-mer are produced.

Relative Affinity of Exonuclease I for Oligodeoxyribonucleotides

The reaction of exonuclease I with oligodeoxyribonucleotides can be assigned the kinetic scheme (Brody et al., 1986):



A processivity parameter P_n can be defined, for each oligomer generated by nucleotide hydrolysis, as the probability that the

[3] The product distribution for the reaction of exonuclease I with an oligomer has been found to depend not only on the oligomer's structure but also on the salt concentration, the buffer composition, and the temperature (R. Brody, unpublished results).

Table II: Contributions of Specific Bases and Phosphates to the Kinetic Affinity of Exonuclease I for Oligodeoxyribonucleotides[a]

	n									
	14	13	12	11	10	9	8	7	6	5
$k_d[(dT_n)]/k_d[(dT)_{n+1}]$		0.7	1.4	1.8	3.5	5.8	1.1	1.0	2.0	2.9
$k_d[(dA)_n]/k_d[(dA)_{n+1}]$			1.8	5.9	2.7	2.8	0.5	0.9	2.7	3.0
interaction lost[b]		$B_{14}P_{13}$	$B_{13}P_{12}$	$B_{12}P_{11}$	$B_{11}P_{10}$	$B_{10}P_9$	B_9P_8	B_8P_7	B_7P_6	B_6P_5
$k_d[(dT)_n]/k_d[p(dT)_n]$		1.3	1.6	2.5	2.4	1.1	0.7	0.7	0.6	1.0
$k_d[(dA)_n]/k_d[p(dA)_n]$		2.0	1.4	5.1	2.9	0.9	0.7	0.9	1.0	1.4
interaction lost		P_{13}^{2-}	P_{12}^{2-}	P_{11}^{2-}	P_{10}^{2-}	P_9^{2-}	P_8^{2-}	P_7^{2-}	P_6^{2-}	P_5^{2-}
$k_d[MP^{n-3}(dT)_n]/k_d[(dT)_n]$	0.9	1.6	1.0	0.8	1.1	1.3	3.2	4.6		
interaction lost	P_{11}	P_{10}	P_9	P_8	P_7	P_6	P_5	P_4		
$k_d[MP^{n-10}(dT)_n]/k_d[(dT)_n]$[c]	2.6	6.4	10.1	75						
interaction lost	P_4	P_3	P_2	P_1						
$k_d[MP^{n-2,n-3}(dT)_n]/k_d[(dT)_n]$[d]	1.0	1.5	3.1	3.8	3.3	1.6	3.6			
interaction lost	P_{11}, P_{12}	P_{10}, P_{11}	P_9, P_{10}	P_8, P_9	P_7, P_8	P_6, P_7	P_5, P_6			
$k_d[AP^{n-2}(dT)_n]/k_d[(dT)_n]$	0.8	0.8	0.6	1.0	2.0	3.4	4.8	5.6		
interaction lost	B_{12}	B_{11}	B_{10}	B_9	B_8	B_7	B_6	B_5		
$k_d[AP^{n-9}(dT)_n]/k_d[(dT)_n]$	1.7	1.5	3.1	28						
interaction lost	B_5	B_4	B_3	B_2						

[a] The k_d values in this table are averages of the k_d values calculated from the individual product distributions used to generate Table I (between 3 and 5 separate reactions and analyses). Ratios that are underlined were found by the *t* test to be different from 1.0 at a 5% significance level. For each entry, the starting materials have a value of $n = 16$ (Table I). [b] "Interaction lost" refers to the differences in the base and phosphate groups of the two oligomers whose dissociation constants are being compared. B_n refers to a nucleotide base in the *n*th base position from the 3′-terminus. P_n refers to a phosphodiester monoanion and P_n^{2-} refers to a 5′-phosphomonoester dianion in the *n*th phosphate position from the 3′-terminus. [c] The results for $MP^6(dT)_{16}$ diastereomer B were used in this table as diastereomer B had a higher affinity for exonuclease I. [d] The results for $MP^{13,14}(dT)_{16}$ diastereomers C and D were used in this table because of their higher affinities for exonuclease I.

enzyme will translocate and hydrolyze the new 3′-terminal phosphodiester rather than dissociate (Brody et al., 1986; McClure & Chow, 1980):

$$P_n = k_h/(k_h + k_d)$$

This equation can be rearranged to express the dissociation constant k_d, in terms of P_n and the rate constant for hydrolysis, k_h (Brody et al., 1986). P_n can be calculated from the data in Table I (Brody et al., 1986).

The rate constant for nucleotide hydrolysis has been shown to be approximately independent of oligomer size for deoxyadenylate 900-mers through tetramers (Brody et al., 1986). Earlier work with thymidylates has shown the hydrolysis rate to be the same for a trimer and a pentamer (Lehman & Nussbaum, 1964). The dissociation constant ratios given in Table II are calculated with the assumption that k_h is constant within the deoxyadenylate and thymidylate series of oligomers. As most of the comparisons in Table II are either between homologues that differ by one nucleotide unit or between oligomers of the same size that have a structural difference in a position remote from the site of nucleotide hydrolysis, the assumption is reasonable. The k_h values cancel when the ratios are taken, and the relative dissociation constants can be calculated from the P_n values. The rate constant for hydrolysis cannot be assumed to be constant if a modification is made in one of the first three nucleotides from the reactive 3′-terminus.

The ratios of dissociation rate constants for the (dT) and (dA) homologue series (Table II; $k_d[(dT)_n]/k_d[(dT)_{n+1}]$ and $k_d[(dA)_n]/k_d[(dA)_{n+1}]$) reflect the effects of single nucleotide units on the rate constant for enzyme–oligomer dissociation during processive hydrolysis. As an example, the results for the (dT) series when *n* is equal to 9 indicates that exonuclease I dissociates from $(dT)_9$ 5.8 times faster than it dissociates from $(dT)_{10}$ (Table II). The difference between $(dT)_9$ and $(dT)_{10}$ is a single nucleotide with a base in the 10th nucleoside position and a phosphodiester in the 9th phosphate position from the 3′-terminus. The loss of the interaction between this nucleotide unit, called $B_{10}P_9$ in Table II, and exonuclease I accounts for the increase in the rate at which the 9-mer dissociates from the enzyme. Although changes in the dissociation rates are sometimes referred to in the text as changes in enzyme–oligomer binding, it must be emphasized that what is being measured is the kinetic affinity between the enzyme and an intermediate generated during processive hydrolysis.

The remaining entries in Table II are ratios of the dissociation constants for oligodeoxyribonucleotides pairs that differ by a chemical modification in a specific position. The chemical changes are the addition of a 5′-terminal phosphate, substitution of one or two methylphosphonate linkages in specified positions, and removal of a nucleoside base in a specified position. The "interaction lost" that is associated with these changes (Table II) are a 5′-phosphate dianion (P^{2-}), a phosphodiester monoanion (P), and a nucleoside base (B).

The ratios in Table II are greater than 1 if the absence of a particular nucleotide unit, charged phosphate species, or nucleoside base results in an oligodeoxyribonucleotide that has a lower affinity for the enzyme. The ratios will be equal to 1 if the modified group has no effect on binding and will be less than 1 if the modification actually increases the affinity of the oligomer for the enzyme.

The ratios of dissociation constants that are significantly different from 1.0 (*t* test at a 5% significance level) are underlined in Table II. The determination that a pair of k_d values are not equal depends both on the difference between their mean values and on the variance of the data. In general, the variance was largest with the small peaks for the 11-mer through the 14-mer. A factor of 2 is the approximate difference between two k_d values needed for statistical significance in our assay.

Discussion

In the first part of this study, the dissociation constants for oligodeoxyribonucleotide homologues that differ by a single nucleotide unit [Figure 1; $(dT)_n$ vs $(dT)_{n+1}$ and $(dA)_n$ vs $(dA)_{n+1}$] have been compared. In cases where the lower homologue binds less tightly to exonuclease I, the lost nucleotide

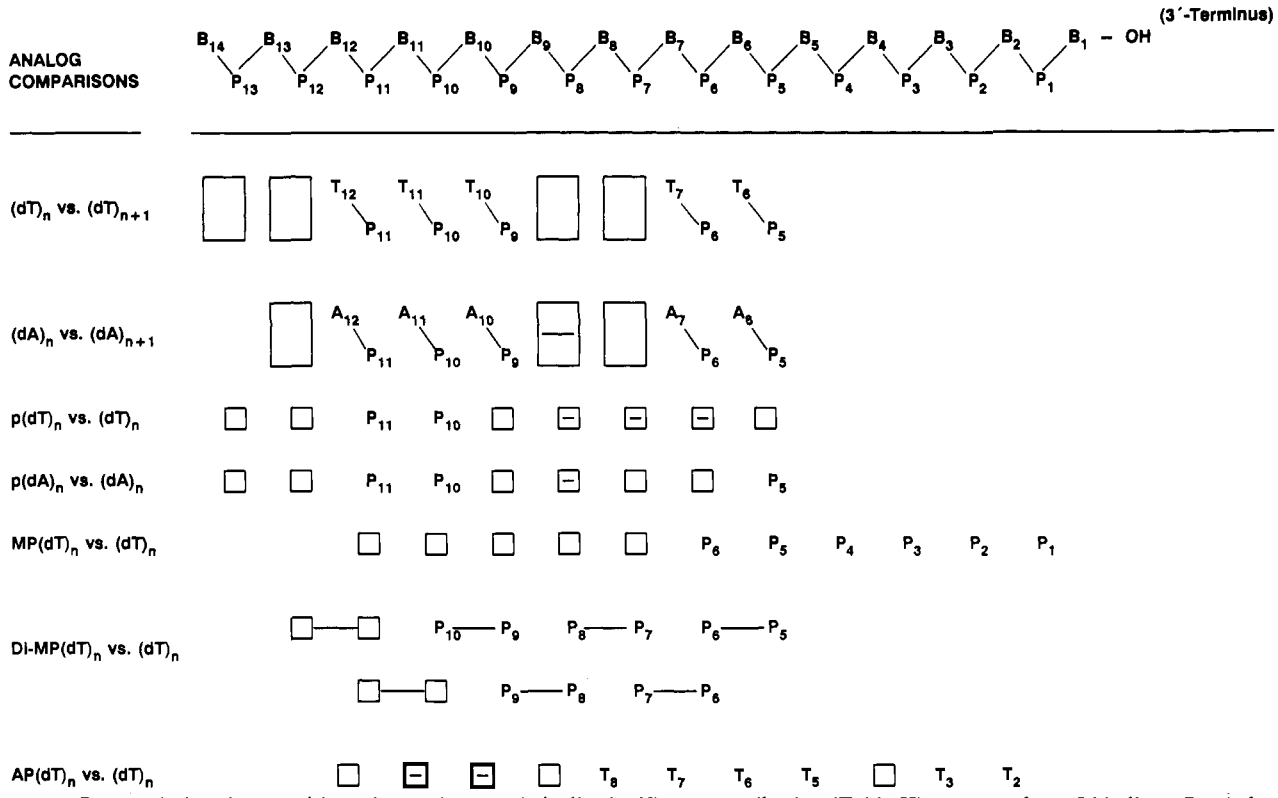


FIGURE 1: Base and phosphate positions that make a statistically significant contribution (Table II) to exonuclease I binding. Symbols: A_n, T_n, adenine (A_n) and thymine (T_n) base positions that contribute to the binding of an analogue to exonuclease I; P_n, phosphodiester positions (P_n) that contribute to the binding of an analogue to exonuclease I; □, base and phosphodiester positions that do not affect the binding of an analogue to exonuclease I; ⊟, base and phosphodiester positions that have a negative effect on the binding of an analogue to exonuclease I; P_n–P_{n-1}, adjacent methylphosphonate positions on the same oligomer that contribute to binding; □—□, adjacent methylphosphonate positions on the same oligomer that do not contribute to binding.

position is postulated to have a direct interaction with the enzyme. Other possible explanations for reduced binding, such as negative steric interactions or conformational effects, are unlikely as the conformations of an unmodified oligomer and its next lower homologue are expected to be very similar. This is particularly the case with the oligothymidylates as these oligomers have very little local structure (Cassani & Bollum, 1969; Bloomfield et al., 1974).

These comparisons have led to a refinement of the positions of the two regions of an oligodeoxyribonucleotide that interact with exonuclease I (Brody et al., 1986). The "anchor site" contains nucleotide units in positions $B_{12}P_{11}$ through $B_{10}P_9$, and the "extended catalytic site" contains residues B_7P_6 and B_6P_5 (Figure 1). Evidence from the analogue study will be presented that indicates that the extended active site also includes nucleotides from B_6P_5 to the 3′-terminus. The region between the two binding sites, B_9P_8 and B_8P_7, does not appear to interact with the enzyme.

A single nucleotide unit, dA_9P_8, has a significant negative effect on binding (Figure 1). Thus $(dA)_9$ binds to the enzyme less tightly than does $(dA)_8$. This negative contribution to binding is not seen in the case of T_9P_8, an indication that the interference is due to the adenine base moiety in the 9th nucleoside position.

In the second part of this study, an attempt has been made to resolve the interaction between oligodeoxyribonucleotides and exonuclease I into contributions from specific nucleoside bases and phosphodiester monoanions. This has been done via reaction of the enzyme with analogues that contain base or phosphate modifications in defined positions. If two oligomers have the same dissociation constant, then the structural difference between them is not important for interaction with the enzyme. Should modification of a specific base or phosphate result in an increased dissociation rate, then a role will be postulated for that position in enzyme binding.

Two additional explanations must also be considered when a modified oligodeoxyribonucleotide displays reduced binding. First, the modified position could interfere with binding via a negative steric or charge interaction. Second, the modification could cause a change in the conformation of the enzyme-bound oligomer so that an interaction distant from the point of modification is disrupted. These possibilities will be discussed for each oligodeoxyribonucleotide comparison. Since in no case can a steric or conformational effect by totally ruled out, the analogue results will be most convincing when the structural change does not affect binding.

The effect of nucleotide modifications in the anchor site will be considered first. Removal of a base moiety in positions B_{12} to B_9 does not decrease the affinity of exonuclease I for an oligomer [Figure 1; $AP(dT)_n$ vs $(dT)_n$]. Rather, the loss of a base in positions B_{11} and B_{10} actually results in slightly increased binding. Exonuclease I, therefore, does not interact with nucleoside bases at the anchor site but instead most likely binds to the phosphodiester monoanions in this site. This hypothesis is supported by results from the 5′-phosphate series [Figure 1; $p(dT)_n$ vs $(dT)_n$ and $p(dA)_n$ vs $(dA)_n$]. In both the (dA) and (dT) oligomers, the presence of a phosphate dianion in anchor positions P_{11} and P_{10} increases the affinity of the enzyme for the oligomer.

In some positions the presence of a 5′-phosphate dianion either does not affect or actually reduces binding (Figure 1). The reduction in binding may be due to conformational changes that occur upon 5′-phosphorylation (Cozzone & Jardetsky, 1976; Bower et al., 1987b). Since a negative conformational effect could cancel out a positive charge interaction, no firm conclusions can be made from these results.

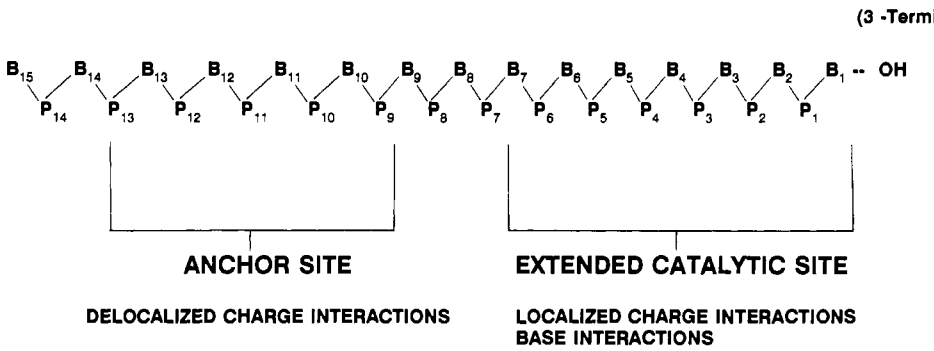


FIGURE 2: Schematic model of oligodeoxyribonucleic acid binding sites for exonuclease I.

In the oligothymidylate and oligodeoxyadenylate series, the loss of nucleotide units $B_{12}P_{11}$, $B_{11}P_{10}$, and $B_{10}P_9$ results in successive decreases in binding (Figure 1). This has been attributed to the loss of 5′-terminal phosphodiester monoanions in positions P_{11}, P_{10}, and P_9. Loss of the same anionic charges in the third phosphate from the 5′-terminus, via methylphosphonate substitution in P_{11}, P_{10}, and P_9 [Figure 1; MP-$(dT)_n$ vs $(dT)_n$], does not affect binding. The enzyme can apparently satisfy its binding requirements by interacting with the two anions that are 5′ of the methylphosphonate group. The ability of the enzyme to bind to alternative positions has been further investigated with the bis(methylphosphonates) [Figure 1; di-MP$(dT)_n$ vs $(dT)_n$]. In this series there is a single phosphodiester monoanion on the 5′-side of the two successive uncharged methylphosphonates. The loss of two charges in P_{12}–P_{11} or P_{11}–P_{10} does not lead to reduced binding (Figure 1), presumably because the enzyme can interact with the anion in P_{13} or P_{12}.

The methylphosphonate results indicate that full binding can be achieved if two or three of the five phosphodiesters between P_{13} and P_9 are anionic. The fact that the enzyme can interact equally well with anions in different anchor site positions is consistent with a long-range, delocalized, electrostatic interaction. This type of interaction has been postulated to provide the binding energy between the catabolite gene activator protein and nonspecific sequences of DNA (Weber & Steitz, 1984). Delocalized binding also appears suited for the translocation of a repressor or activator protein along DNA in a search for its specific binding site (von Hippel & Berg, 1989). The translocation of the active site of exonuclease I after nucleotide hydrolysis may be an analogous process.

An alternative explanation for the methylphosphonate results is that the enzyme might be able to form hydrogen bonds with both phosphate monoanions and methylphosphonates in the anchor site. This explanation is unlikely as exonuclease I binds equally well to both mono(methylphosphonate) diastereomers in the anchor site, only one of which should have an oxygen available for hydrogen bonding to the enzyme.

The region between the two binding sites extends from B_9 through P_7 and has been shown by the (dA) and (dT) homologue results not to interact with the enzyme (Figure 1). This conclusion is consistent with the results from the oligodeoxyribonucleotide 5′-phosphates and mono(methylphosphonates) (Figure 1), as the loss of anionic charges in positions P_8 and P_7 does not results in decreased binding. In contrast, the substitution of two methylphosphonates in positions P_8–P_7 and the creation of an abasic site in position B_8 decrease the affinity of exonuclease I for an oligomer. Both the methylphosphonate substitution (Chacko et al., 1983; Han et al., 1990) and the abasic substitution (Kalnick et al., 1988), however, can result in conformational changes that may account for the reduced binding.

The final binding domain, the extended catalytic site, appears to interact with the enzyme via both nucleoside bases and phosphodiester monoanions. Significant reductions in binding are caused by removal of a base in positions B_7 to B_5 and in positions B_3 to B_2 [Figure 1; $AP(dT)_n$ vs $(dT)_n$]. The results with the $MP^{13}(dT)_{16}$ series indicate that substitution of a methylphosphonate in positions P_6 to P_1 also reduces enzyme binding [Figure 1; MP$(dT)_n$ vs $(dT)_n$].

The loss of bases B_3 and B_2 and phosphodiester monoanions P_2 and P_1, which are adjacent to or part of the catalytic site, may affect the product distribution by reducing the rate of nucleotide hydrolysis. In the other positions, the reduction in binding may be due to a conformational or steric effect rather than to the loss of a specific interaction at the extended catalytic site. This possibility makes detailed analyses of these effects unprofitable beyond the general conclusion that bases and phosphodiester monoanions in the extended active site are essential for optimal binding. This same qualification limits any interpretation of the effect of different methylphosphonate diastereomers on binding.

The affinity of the enzyme for each oligodeoxyribonucleotide appears to be due to the cumulative effect of small interactions along the chain (Table II). Many significant interactions only change the dissociation rate by a factor of 2, and the largest effects in Table II, disregarding those caused by modifications at or near the active site, correspond to a factor of 6. These dissociation rate comparisons have not been converted to free energies of binding as no association rate data have been obtained. If it can be assumed that at least some of the oligomers compared in Table II have similar association rates, then the maximal binding energy provided by a position is on the order of 1 kcal/mol. This is comparable to the contribution of each bound phosphate group to the free energy of interaction between a DNA binding protein and nonspecific DNA sequences under similar low-salt conditions (Revzin & von Hippel, 1977; deHaseth et al., 1977).

Conclusions

The results with the oligodeoxyadenylate and oligothymidylate homologues clearly show that the exonuclease I binding site extends 11 nucleotides from the 3′-terminus. The bis(methylphosphonate) data indicate that nucleotides 12 and 13 may also participate in binding. Long nucleic acid binding sites have been demonstrated in other proteins that bind to single-stranded nucleic acids. The gene 5 protein (Brayer & McPherson, 1984) and the gene 32 protein (Prigodich et al., 1984; Kowalczykowski et al., 1986), for example, bind to approximately 5 and 8 nucleotides, respectively. Long binding sites are also common among double strand specific binding proteins such as the *lac* repressor. This protein remains associated with approximately 12 base pairs as it searches for

its specific target sequence by facilitated diffusion (deHaseth et al., 1977; Revzin & von Hippel, 1977; von Hippel & Berg, 1989).

The nucleic acid binding site can be divided into the two domains shown schematically in Figure 2. The anchor site extends from phosphate positions P_{13} through P_9, with respect to the 3′-terminus, and most likely interacts with the enzyme via delocalized charge interactions. The extended catalytic site stretches from position B_7 to the 3′-terminus and requires both phosphodiester monoanions and base residues for exonuclease I binding. The nucleotides between the domains do not appear to interact with the enzyme.

A complete understanding of the relationship of these two separated binding sites to the processive reaction must await further structural and kinetic studies, but some preliminary proposals can be made. The two key steps in the processive reaction of exonuclease I with oligodeoxyribonucleotides have different binding requirements. Nucleotide hydrolysis requires hydrogen bonds and localized electrostatic interactions in order to fix the labile phosphodiester in the enzyme's active site. This localized binding occurs between exonuclease I and the extended catalytic site. Translocation, on the other hand, requires bonds that can be made and broken easily as the enzyme moves along the oligomer. Long-range electrostatic attractions are postulated to be appropriate for such an interaction (von Hippel, 1989) and can be provided by the anchor site of exonuclease I. The anchor site interaction in this model must only be tight enough to allow the enzyme to translocate a single nucleotide in order to position the new 3′-terminus in the active site. The binding has been shown not to be strong enough to allow the facilitated diffusion of exonuclease I from internal oligodeoxyribonucleotide sites to the 3′-terminus (Brody et al., 1986).

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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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