Noncovalent DNA Binding and the Mechanism of Oxidative DNA Damage by Fecapentaene-12

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The fecapentaenes are a group of mutagenic, polyunsaturated lipids that are produced endogenously in the human gastrointestinal tract. Previous studies show that the fecapentaenes cause oxidative DNA damage, but the chemical mechanisms by which this occurs remain unclear. The data presented here indicate that fecapentaene-12 causes direct oxidative DNA damage via production of the reactive oxygen species $O_2^{\bullet-}$, H_2O_2, and HO•. In addition, evidence is presented indicating that fecapentaene-12 associates noncovalently with duplex DNA. Fecapentaene-12 provides an interesting new example highlighting the potential for hydrophobic long chain hydrocarbons to associate noncovalently with duplex DNA.

Introduction

The fecapentaenes are a class of mutagenic polyunsaturated lipids that are produced endogenously in the human gastrointestinal tract (*1*, *2*). Because these agents may contribute to the occurrence of colon cancer, the nature of the DNA-damage processes underlying their potent mutagenic properties is of interest. It is clear that the fecapentaenes cause molecular oxygen-dependent oxidative DNA damage; however, the chemical mechanisms by which this occurs have been the subject of debate (*1*−*7*). Here, we report the results of studies designed to clarify the mechanisms of direct oxidative DNA damage by fecapentaene-12 (**1**). Our data indicates that **1** causes direct DNA damage via production of the reduced oxygen species ($O_2^{\bullet-}$, H_2O_2, HO•). In addition, we present the first clear evidence indicating that **1** associates noncovalently with duplex DNA.

Experimental Procedures

Materials. Materials were obtained from the following suppliers and were of the highest purity available (≥95% purity): tetrabutylammonium fluoride, potassium carbonate, sodium phosphate, bromophenol blue, sucrose, EDTA, HEPES buffer, and desferal from Aldrich Chemical Co. (Milwaukee, WI); HPLC grade solvents (water, acetonitrile, ethanol, methanol, and diethyl ether) from Fisher (Pittsburgh, PA); ethidium bromide and herring sperm DNA from Roche Molecular Biochemicals (Indianapolis, IN); agarose, from Seakem; catalase, calf thymus DNA, and SOD from Sigma Chemical Co. (St. Louis, MO). The plasmid pGL2BASIC was prepared using standard methods (*8*).

Synthesis of Fecapentaene-12 (1). Compound **1** was prepared via the eight-step synthetic route devised by Nicolaou and co-workers (*9*) except *tert*-butyl dimethylsilyl protecting groups were employed rather than *tert*-butyl diphenylsilyl groups. In our synthesis, the *tert*-butyl dimethylsilyl protecting groups were introduced at the beginning of the synthesis as described by Nicolaou and co-workers for the *tert*-butyl diphenylsilyl protecting group (*9*). The final deprotection reaction was carried out as follows: to a solution of protected **1** (10 mg, 0.021 mmol) in THF (0.5 mL), tetrabutylammonium fluoride (1 M in THF; 300 μL, 0.3 mmol, 15 equiv) was added at 24 °C under nitrogen gas in a round-bottom flask protected from light. The reaction mixture immediately turned red. The mixture was stirred until all starting material was consumed (approximately 10 min), and then degassed diethyl ether was added (15 mL). The organic solution was washed with water (250 μL) and brine (250 μL) and then dried over K_2CO_3 under an atmosphere of nitrogen gas protected from light. The drying agent was filtered from the solution under a nitrogen atmosphere and the ether layer concentrated by passing a stream of nitrogen gas over the solution. The compound was purified by flash-column chromatography under an inert atmosphere in the dark using degassed solvents (1% methanol in ether) to yield a lemon yellow solid (2−3 mg, 32−48%). R_f = 0.35 (2% methanol in ether). The NMR spectrum of **1** matched that reported previously (*10*).

Cleavage of Plasmid DNA by 1 or Vitamin A. Compound **1** and vitamin A were delivered as stock solutions (0.5−5 mM) in 100% ethanol. Samples of **1** used in the DNA-cleavage experiments reported here were at approximately 95% pure at the beginning of the experiments. Compound **1** decomposes more rapidly in concentrated solutions. The half-life of a 200 μM solution of **1** under these experimental conditions is about 15 h. All reactions were conducted in 50 mM sodium phosphate (pH 7.0) containing 10% acetonitrile by volume and employed 30 μM (bp) plasmid in 50 μL final volume. In a typical assay, an appropriate volume of **1** or vitamin A stock solution in ethanol was placed in a microcentrifuge tube (500 μL) and evaporated to dryness under a stream of nitrogen gas. Acetonitrile (5 μL) and sodium phosphate buffer (5 μL of a 500 mM pH 7.0 stock solution in water), followed by water (39 μL), were added, and the mixture was agitated on a vortex mixer for 20 s. Finally, plasmid DNA (1 μL of a 1 mg/mL solution) was added, and the mixture was gently vortexed again. Samples were incubated in the dark for 15 h at 37 °C. Following incubation, glycerol loading buffer (7 μL) containing 0.25% bromophenol blue and 40% sucrose was added, and the mixture was vortexed. The resulting mixture was loaded on a 0.9% agarose gel. The gel was electrophoresed at 80 V for approximately 4 h and then stained in an aqueous ethidium bromide solution (0.2 μg/mL) for 6−8 h. The DNA in the gel was then visualized by UV-transillumination, and the data were documented using an Alpha Innotech IS1000 digital imaging system, with Alphaimager software. Mechanistic experiments containing additives such as desferal, ethanol, methanol, mannitol, superoxide dismutase (SOD), and catalase were performed in an identical manner.

Plasmid DNA Cleavage by Partially Degraded 1. Microcentrifuge tubes containing various amounts of neat **1** were prepared as described above. Acetonitrile or ethanol (12.5 μL) and sodium phosphate buffer (5 μL of a 500 mM stock solution in water), followed by water (31.5 μL), were added, and the mixture was agitated on a vortex mixer for 20 s. Before the addition of plasmid DNA, the mixtures were preincubated for 5 h at 37 °C. Plasmid DNA (1 μL of a 1 mg/mL solution) was added with gentle vortex mixing, and the cleavage reactions were incubated in the dark for

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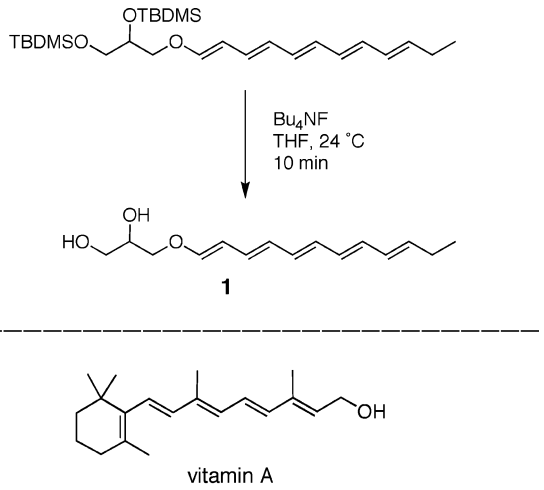
Scheme 1



15 h at 37 °C. Analysis of DNA damage was performed as described above.

Displacement of Ethidium from Duplex DNA by 1. A mixture of ethidium bromide and calf thymus DNA in aqueous buffer was placed in a 400 μL quartz fluorescence cuvette (final concentrations: DNA, 4 μM (bp); ethidium bromide, 2 μM; HEPES buffer, 10 mM pH 7; EDTA, 0.5 mM; sodium chloride, 8 mM; acetonitrile, 30% v/v). The sample was analyzed using a Photon International Technology fluorimeter using the following settings: excitation wavelength 545 nm, emission wavelength 596 nm, optical slits at 8 nm, lamp power 75 W, detector amplification at 1000 V, and a light-integration time of 0.1 s. The fluorimeter was zeroed on a solution containing ethidium without DNA. Aliquots of a concentrated solution of **1** (2−5 μL of a 5 mM solution in ethanol) were added, and changes in fluorescence emission of DNA-bound ethidium were monitored. The readings were corrected for dilution. As controls, the effect of pure ethanol addition on the fluorescence of the DNA−ethidium bromide complex and the fluorescence of **1** alone were measured. The data were used to estimate the binding constant of **1** with duplex DNA using the general approach described by Baguley et al. (*11*). These calculations utilized a binding constant of 9.5×10^6 M^{-1} for association of ethidium bromide with calf thymus DNA as reported previously (*12*).

Effect of Added Duplex DNA on the UV−Vis Spectrum of 1. A solution of **1** in sodium phosphate buffer was placed in a UV−vis cuvette (final concentrations: **1**, 40 μM; sodium phosphate, 50 mM, pH 7; acetonitrile, 25 vol %). To this mixture, aliquots of a solution containing herring sperm DNA (5 mM bp) and **1** (40 μM) in sodium phosphate buffer (50 mM, pH 7) and acetonitrile (25 vol %) were added, and the resulting mixtures were analyzed by UV−vis spectroscopy. The aliquots of added DNA contained fecapentaene to ensure that changes in the spectrum were not due to dilution. The DNA titration experiment was complete within 25 min. A control experiment was carried out to show that the changes in the UV−vis spectrum are not due to chemical degradation of **1** or reaction of the compound with DNA over the course of the experiment. Specifically, the control showed that the degradation of **1** in the presence of DNA (10 mM bp) over the course of 25 min yields relatively small changes in the UV−vis spectrum compared to those seen in the DNA titration shown in Figure 4.

Results and Discussion

We prepared compound **1** via the eight-step synthetic route devised by Nicolaou and co-workers (*9*), with one minor modification. We employed *tert*-butyl dimethylsilyl groups to protect the alcohol moieties rather than the *tert*-butyl diphenylsilyl groups used previously. This approach decreased the reaction time required for the final deprotection step, thus, minimizing degradation of the unstable product (**1**, Scheme 1).

Table 1. DNA Damage by Fecapentaene-12 (1), Vitamin A, and the Effect of Various Additives[a]

reaction and additive	% nicked DNA	*S*-value
DNA alone	11	0.12 ± 0.02
DNA + 200 μM **1** (std rxn)	72	1.28 ± 0.06
std rxn in chelexed buffer	50	0.71 ± 0.02
std rxn in degassed buffer	31	0.37 ± 0.03
std rxn + 200 mM methanol	34	0.42 ± 0.02
std rxn + 200 mM ethanol	27	0.31 ± 0.01
std rxn + 100 mM mannitol	34	0.42 ± 0.01
std rxn + 10 mM desferal	32	0.39 ± 0.03
std rxn + 100 μg/mL SOD	52	0.74 ± 0.01
std rxn + 100 μg/mL catalase	40	0.51 ± 0.03
DNA + 300 μM vitamin A (std rxn)	49	0.67 ± 0.02
std rxn in degassed buffer	21	0.23 ± 0.02
std rxn + 200 mM methanol	27	0.31 ± 0.02
std rxn + 200 mM ethanol	23	0.26 ± 0.02
std rxn + 100 mM mannitol	25	0.29 ± 0.0
std rxn + 10 mM desferal	27	0.31 ± 0.0
std rxn + 100 μg/mL SOD	30	0.35 ± 0.1
std rxn + 100 μg/mL catalase	32	0.39 ± 0.01

[a] Supercoiled plasmid DNA (30 μM bp) was incubated for 15 h at 37 °C with various concentrations of **1** or vitamin A in sodium phosphate buffer (50 mM) containing 10% acetonitrile (by volume). Agarose gel electrophoresis was performed as described previously (*13*). The *S*-value represents the mean number of strand breaks per plasmid molecule and is calculated using the equation $S = -\ln f_I$, where f_I is the fraction of plasmid in a given lane that is present as uncut, form I DNA. The relationship between % nicked plasmid and the actual number of strand breaks is nonlinear. The *S*-value provides a direct assessment of the amount of strand cleavage that has occurred in a given reaction. Standard error in these measurements is less than 10%.

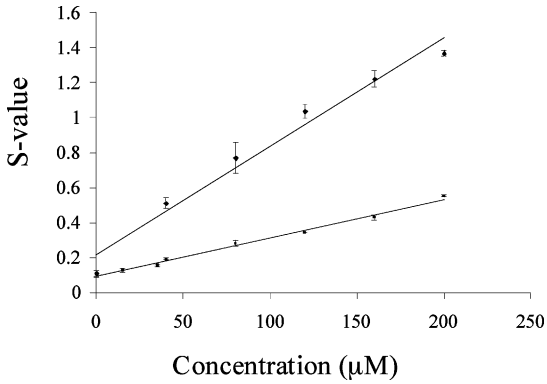


Figure 1. DNA-cleavage by fecapentaene-12 (**1**, upper line) and vitamin A (lower line). Various concentrations of **1** or vitamin A were incubated with plasmid DNA, and strand cleavage was analyzed as described in the legend of Table 1. The *S*-value is the mean number of strand breaks per plasmid molecule and is calculated using the following equation: $S = -\ln f_I$, where f_I is the fraction of uncut, form I DNA remaining. The standard error in these measurements is approximately ±10%.

We employed a plasmid-based assay to characterize the DNA-cleaving properties of **1**. In this assay, single-strand cleavage converts supercoiled double-stranded plasmid (form I) into the open circular form (II) (*13*). The two forms of plasmid DNA are then separated using agarose gel electrophoresis and visualized by staining with ethidium bromide. We find that incubation of **1** with plasmid DNA leads to the production of direct single-strand breaks (Table 1 and Figure 1). We then carried out a series of experiments designed to examine whether DNA strand cleavage by **1** occurs via the cascade of reactions involving reduced oxygen species shown in eqs 1−4. We find that DNA cleavage by **1** can be inhibited by addition of typical radical scavengers such as methanol, ethanol, and mannitol, by the hydrogen peroxide-destroying enzyme catalase, and by the chelator of adventitious trace metals, desferal, which is known to suppress the metal-dependent Fenton reaction (eq 3, where M^{n+} is a transition metal such as Fe^{2+}) (*14*). Chelex treatment of the buffer to decrease the levels of adventitous trace metals results in decreased DNA cleavage (Table 1). Addition of the enzyme

superoxide dismutase (SOD) also inhibits DNA strand cleavage, presumably by preventing superoxide-dependent reduction of the trace metals (eq 4) that are required for the Fenton reaction (eq 3). This effect of SOD is observed in the context of in vitro systems where alternative metal-reducing agents such as ascorbate or thiols are not present (*14*). Freeze−pump−thaw degassing of the assay mixture to remove dissolved oxygen causes a significant decrease in DNA cleavage yield (Table 1).

Taken together, the findings presented in Table 1 are consistent with a mechanism where **1** causes DNA strand cleavage via the well-known (*14*) cascade of reactions shown in eqs 2−4, in which hydroxyl radical is produced as the ultimate DNA-cleaving agent. Hydroxyl radical is a well-known DNA-damaging agent (*15*−*18*). The initial steps in this overall process likely involve oxidation of the polyene framework in **1** to yield superoxide radical ($O_2^{\bullet-}$) and the resonance-stabilized polyene radical cation (*19*, *20*). Subsequent reactions involved in the aerobic degradation of **1** are poorly defined and undoubtedly complex (*20*, *21*). While much recent work has focused on peroxidase-dependent (*22*) production of superoxide by **1** (*2*, *5*, *6*), our results demonstrate the potential of this compound to *directly* generate DNA-damaging reactive oxygen species from molecular oxygen. A fecapentaene radical or fecapentaene-derived peroxyl radicals may have the potential to cause additional types of DNA damage, but our studies offer no evidence for (or against) this possibility.

$$\mathbf{1} + O_2 \rightarrow O_2^{\bullet-} + \mathbf{1}^{\bullet+} \quad (1)$$

$$O_2^{\bullet-} + HO_2\bullet \rightarrow H_2O_2 + O_2 \quad (2)$$

$$H_2O_2 + M^{n+} \rightarrow HO\bullet + HO^- + M^{(n+1)+} \quad (3)$$

$$O_2^{\bullet-} + M^{(n+1)+} \rightarrow O_2 + M^{n+} \quad (4)$$

Others have suggested (*5*, *6*) that the fecapentaenes may produce the DNA-damaging species singlet oxygen and hydroxyl radical via the Haber−Weiss reaction (eq 5). However, this seems unlikely in light of studies from several groups showing that this general pathway essentially does not occur under physiological conditions (*23*−*26*). In fact, the evidence supporting the generation of singlet oxygen by the fecapentaenes is not overwhelming. For example, inhibition of DNA damage by sodium azide has been taken as a sign of singlet oxygen involvement (*6*). However, while sodium azide does, indeed, quench singlet oxygen (*27*−*29*), it also effectively scavenges hydroxyl radical, with a rate constant of 1.2×10^{10} M^{-1} s^{-1} (*30*). Furthermore, reactions of fecapentaene with DNA have been carried out in D_2O, which increases the lifetime of singlet oxygen, but only minimal increases in damage yields were observed (*5*). In cases where singlet oxygen is clearly involved, large increases in the yields of DNA damage typically are observed when the reaction is performed in D_2O (*31*). Rather than invoking singlet oxygen, it seems likely that much of the published data regarding oxidative DNA damage by the fecapentaenes (both enzyme-dependent and spontaneous) can be rationalized in terms of the chemical reactions shown in eqs 1−3.

$$H_2O_2 + O_2^{\bullet-} \rightarrow HO\bullet + {}^1O_2 + HO^- \quad (5)$$

We compared DNA damage by **1** to that by another naturally occurring polyene, vitamin A. Previous work has indicated that autoxidation of vitamin A produces superoxide radical under physiologically relevant conditions (*32*). Accordingly, we observe that vitamin A causes direct strand cleavage in the plasmid assay utilized here (Table 1, Figure 1). The effect exerted by various additives on the yields of DNA cleavage by vitamin A mirror their effects on cleavage by **1** (Table 1). These results support the idea that both **1** and vitamin A cause DNA damage via the reactive oxygen species shown in eqs 2−4.

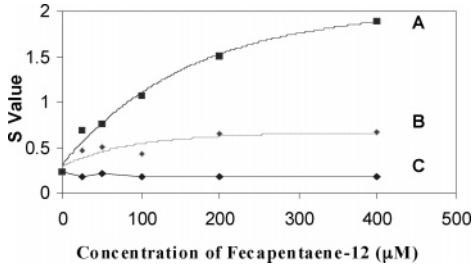


Figure 2. Comparison of DNA cleavage by fecapentaene-12 (**1**) and preincubated (degraded) fecapentaene-12. DNA-cleavage assays were conducted as described in the legend of Table 1. The *S*-value is the mean number of strand breaks per plasmid molecule and is calculated using the following equation: $S = -\ln f_I$, where f_I is the fraction of uncut, form I DNA remaining. Curve A, **1** (without any preincubation)l; curve B, **1** preincubated in 50 mM sodium phosphate buffer (pH 7.0) containing 30% acetonitrile for 5 h at 37 °C; curve C, **1** preincubated in 50 mM sodium phosphate buffer (pH 7.0) containing 30% ethanol for 5 h. The standard error in these measurements is approximately ±10%.

A number of reports have detailed the instability of the fecapentaenes (*1*, *2*, *33*, *34*). We confirm that **1** is, indeed, unstable and further demonstrate that decomposition yields products with vastly decreased ability to cause DNA strand breaks (Figure 2). For example, following preincubation of **1** in ethanol-containing buffer for 5 h, essentially no DNA-cleaving activity remains (Figure 2). Earlier work has shown that the mutagenic activity of the fecapentaenes diminishes upon preincubation (*34*). It is noteworthy that the decomposition of **1** in ethanol-containing buffer is more rapid than in the acetonitrile-containing buffers used in our typical DNA damage experiments. The half-life of **1** (200 μM) in the 10% acetonitrile buffer system used for the DNA cleavage experiments is approximately 15 h.

Finally, we investigated the ability of **1** to associate noncovalently with duplex DNA. This is an important consideration because it is well-known that the DNA-damaging potential of agents that generate reactive oxygen species can be enhanced by noncovalent association with the duplex DNA (*35*). The grooves of the double helix are hydrophobic (*36*−*39*), and there are some intriguing indications that long-chain hydrocarbons can associate noncovalently with duplex DNA (*40*−*42*). To date, however, this mode of DNA binding has not been well-characterized.

To explore whether **1** associates noncovalently with DNA, we used the well-known ethidium displacement assay (*11*, *12*). This technique employs fluorescence spectroscopy to quantitatively monitor the ability of a putative DNA-binding agent to displace intercalated ethidium from the DNA double helix. A wide variety of DNA-binding molecules including intercalators, groove binders, and polycations are able to displace ethidium in this type of assay (*11*, *12*, *43*−*46*).

We find that titration of the ethidium−DNA complex with micromolar concentrations of **1** causes displacement of the ethidium fluorophore from the duplex (Figure 3). Calculations (*11*, *12*) based upon the concentration of **1** required to displace 50% of the DNA-bound ethidium yield an association constant of $(6.5 \pm 1.1) \times 10^4$ M^{-1} for the noncovalent association of **1**

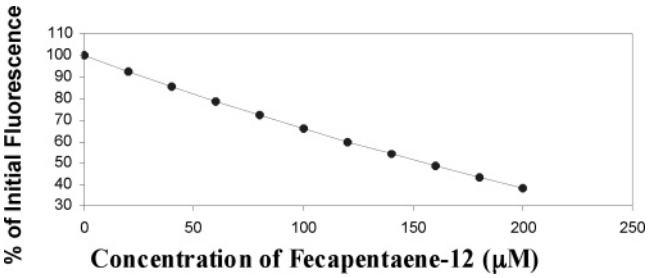


Figure 3. Ethidium displacement by fecapentaene-12 (**1**). A solution of 4 μM calf thymus DNA and 2 μM ethidium bromide in 10 mM HEPES buffer (pH 7.0), 0.5 mM EDTA, and 8 mM NaCl containing 30% acetonitrile was titrated with a stock solution of **1** in ethanol. The displacement of ethidium from the DNA duplex was followed by measuring the decrease in the fluorescence of DNA-bound ethidium using excitation at 545 nm while monitoring emission at 596 nm. Control experiments demonstrated that the **1**−DNA complex is not fluorescent, that addition of the cosolvent (ethanol) alone does not cause a significant decrease in fluorescence of the ethidium−DNA complex, and that **1** does not significantly alter the fluorescence of free ethidium. In contrast to **1**, vitamin A does *not* displace ethidium from duplex DNA under identical conditions.

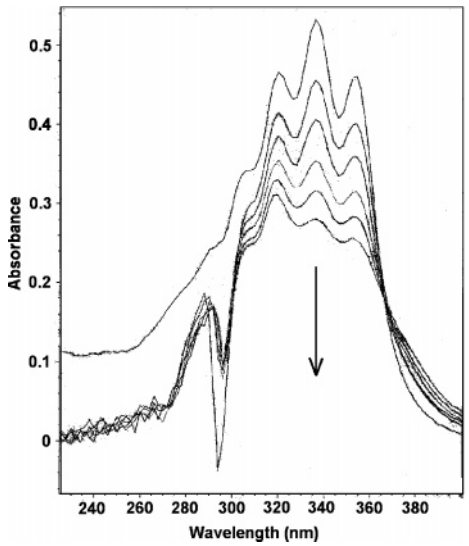


Figure 4. Changes in the UV−vis spectra of **1** with increasing concentration duplex DNA. Aliquots of herring sperm DNA were added to a solution of **1** (40 μM) in 50 mM sodium phosphate buffer (pH 7.0, containing 25% acetonitrile). The concentration of **1** was maintained at 40 μM for each measurement. The DNA concentrations for each curve (from top to bottom) were 0, 1, 2, 4, 6, and 8 mM (bp).

with duplex DNA. These calculations assume that **1** and ethidium bromide compete for the same binding sites on DNA and most likely overestimate the binding constant. Nonetheless, this measurement offers a useful estimate regarding the affinity of **1** for the double helix.

We further confirmed the noncovalent interaction of **1** with duplex DNA using a UV−vis spectroscopic assay. The UV−vis spectra of organic ligands are often perturbed upon association with DNA (*12*, *47*, *48*). We find that titration of **1** with duplex DNA yields a concentration-dependent change in the UV−vis spectrum of **1**. Specifically, addition of DNA to **1** causes a 40% decrease in the absorbance, without any significant change in the absorbance maxima. This type of hypochromism without a concurrent red shift or blue shift in the spectrum has been reported for other agents that associate with the duplex via a groove-binding mode (*49*). A control experiment indicates that the changes observed in the UV−vis spectrum of **1** during the DNA titration are not due to decomposition of the compound. While decomposition yields similar changes in the spectrum of **1**, the control reaction shows that incubation of **1** with 10 mM bp DNA for 25 min yields relatively small (∼10%) changes in the UV−vis spectrum. The observed changes in the UV−vis spectrum of **1** also cannot be ascribed to a salt effect resulting from the addition of DNA−phosphate residues, as we find that addition of analogous amounts of sodium phosphate buffer do not cause a marked change in the compound's spectrum. Perturbation of the UV−vis spectrum of **1** upon addition DNA has been observed previously (*50*), although the exact experimental conditions were not described.

Conclusions

In summary, we find that fecapentaene-12 (**1**) causes oxidative DNA damage through the direct production of the reactive oxygen species $O_2^{\bullet -}$, H_2O_2, and HO•. Oxidative DNA damage arising through this general pathway (*16*, *17*, *51*) clearly could contribute to the potent mutagenicity of the fecapentaenes. In addition, our results indicate that **1** interacts noncovalently with duplex DNA. Thus, the fecapentaenes provide an interesting new example highlighting the potential for long chain hydrocarbons to associate noncovalently with double-helical DNA.

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