Volume 15 Number 3 1987 Nucleic Acids Research

The isolation and characterisation of a new type of dimeric adenine photoproduct in UV-irradiated deoxyadenylates

Shiv Kumar[1], Narain D.Sharma[1], R.Jeremy H.Davies[1*], Douglas W.Phillipson[2] and James A.McCloskey[2]

[1]Biochemistry Department, Medical Biology Centre, Queen's University, Belfast BT9 7BL, Northern Ireland and [2]Departments of Medicinal Chemistry and Biochemistry, University of Utah, Salt Lake City, UT 84112, USA

Received November 5, 1986; Revised and Accepted December 31, 1986

ABSTRACT

A new type of dimeric adenine photoproduct has been isolated from d(ApA) irradiated at 254 nm in neutral aqueous solution. It is formed in comparable amounts to another, quite distinct, adenine photoproduct first described by Pörschke (J. Am. Chem. Soc. (1973), 95, 8440-8446). Results from high resolution mass spectrometry and ^{1}H NMR indicate that the new photoproduct comprises a mixture of two stereoisomers whose formation involves covalent coupling of the adenine bases in d(ApA) and concomitant incorporation of the elements of one molecule of water. The photoproduct is degraded specifically by acid to 4,6-diamino-5-guanidinopyrimidine (DGPY) whose identity has been confirmed by independent chemical synthesis. Formation of the new photoproduct in UV-irradiated $d(pA)_2$ and poly(dA), but not poly(rA), has been demonstrated by assaying their acid hydrolysates for the presence of DGPY. The properties of the photoproduct are consistent with it being generated by the hydrolytic fission of an azetidine photoadduct in which the N(7) and C(8) atoms of the 5'-adenine in d(ApA) are linked respectively to the C(6) and C(5) positions of the 3'-adenine.

INTRODUCTION

Far ultraviolet (UV) radiation induces a variety of photoreactions in DNA which predominantly involve the pyrimidine bases thymine and cytosine[1-3]. For many years there was no convincing evidence for the formation of any photoproducts derived from the purine bases adenine and guanine apart from their photochemical hydroxyalkylation at C(8) which occurs when DNA is irradiated in the presence of 2-propanol[4,5]. Recently, however, we have demonstrated[6,7] that the adjacent thymine and adenine bases in T-A doublets give rise to a mixed pyrimidine-purine photoadduct when native or denatured DNA is irradiated at 254 nm. Other recent studies[8,9], where gel sequencing techniques have been used to locate the position of photolesions in defined segments of DNA, also provide clear evidence for the existence of purine photoproducts though their identities are unknown. These findings have emerged some ten years since Pörschke first reported[10,11] the occurrence of a specific photoreaction, with a relatively

high quantum yield (1 - 2.5 x 10^{-3} mol einstein^{-1}), in dimers and higher polymers of deoxyadenylic acid. Although 3'- and 5'- dAMP, like other monomeric purine derivatives, are extremely resistant to photochemical alteration[4,12], Pörschke observed characteristic spectroscopic changes indicative of photoproduct formation in samples of oligo(dA) and poly(dA) irradiated at 248 nm. Interestingly, the photoreaction does not occur in riboadenylates[10,13]. In the case of $d(pA)_2$, a discrete photoproduct was isolated by ion exchange chromatography which, primarily on the basis of its optical properties, was postulated to arise by covalent linking of the two adenine bases in the dinucleotide[11]. In 1986, Gasparro and Fresco[14] published an extensive study of the fluorescence properties exhibited by UV-irradiated oligo- and poly-nucleotides containing adenine. They concluded that the photoproduct described by Pörschke contains a dehydrodimer of adenine in which the two bases are linked together through their respective C(8) positions as occurs in the model compound 8-(8-adenosyl)adenosine[15].

In an attempt to advance the structural characterisation of this dimeric adenine photoproduct we examined the photochemistry of the deoxydinucleoside monophosphate d(ApA) when it is irradiated at 254 nm in neutral aqueous solution. Pörschke noted photoproduct formation in d(ApA)[13] and also that the photoreaction in $d(pA)_2$ occurs almost as readily on irradiation at 260 nm as at 248 nm[11]. When reversed-phase HPLC was used to analyse the irradiation products of d(ApA) we found unexpectedly (see Fig.1) that comparable amounts of <u>two</u> major photoproducts are formed. One of them corresponds in its spectroscopic and chromatographic properties to the type of photoproduct isolated previously by Pörschke. The other, however, has not been detected heretofore. In this paper, we describe the isolation and spectroscopic properties of this new d(ApA) photoproduct as well as its specific degradation by acid to 4,6-diamino-5-guanidinopyrimidine (DGPY). By means of the latter reaction, we have demonstrated that the new photoproduct is also formed in UV-irradiated $d(pA)_2$ and poly(dA).

EXPERIMENTAL

Materials

The ammonium salt of d(ApA), the potassium salt of poly(rA), 4,5,6-triaminopyrimidine sulphate, cyanamide, trifluoroacetic acid (TFA), 1,3-dimethyluracil, and alkaline phosphatase (from bovine intestine) were obtained from Sigma. Other materials were purchased from commercial suppliers as follows: the sodium salt of poly(dA) and Sephadex G-10 from

Pharmacia; the sodium salt of $d(pA)_2$ from P-L Biochemicals; HPLC grade methanol from May and Baker.

4,5-Diamino-2-guanidinopyrimidine, 4,6-diamino-2-guanidinopyrimidine and 4,5-diamino-6-guanidinopyrimidine were a gift from Dr D.L. Ladd[16].

Methods

UV spectra were recorded with a Unicam SP-800 or Cary 118 spectrophotometer. ^1H and ^{13}C NMR spectra were obtained with a Bruker WM 250 Fourier transform spectrometer; tetramethylsilane and tetramethylammonium chloride (δ 3.19) were used as internal standards to calibrate spectra run in Me_2SO-d_6 and D_2O respectively.

Electron ionization (EI) mass spectra were recorded with a MAT 731 mass spectrometer operating at 70 eV ionizing energy. The same instrument was used to obtain fast atom bombardment (FAB) mass spectra from samples dissolved in a glycerol matrix; ionization was induced with a 6 keV Xe beam produced by an Ion Tech FAB 11N ion source. Exact mass values were determined by peak matching, at 10000 resolution, using appropriate perfluoroalkane ions as the EI reference masses and glycerol cluster ions as the FAB internal standard. Tandem mass spectra were recorded at VG Analytical, Manchester, England, using a VG 7070EQ mass spectrometer operated at 30 eV collision energy with FAB ionization. Deuterium exchange of active hydrogens prior to vaporization was achieved by dissolving the sample in D_2O and evaporating the solvent under vacuum twice before transferring the sample to the FAB probe tip which contained [hydroxy-$^2H_3$]glycerol as the matrix[17]. Trimethylsilyl (TMS) derivatives for EI mass spectrometry were prepared by heating approximately 10 µg of vacuum-dried material with N,O-bis(trimethylsilyl)acetamide, trimethylchlorosilane and pyridine (100:1:50) in a total volume of 10 µl, in a sealed capillary tube for 1 h at 100°C.

High voltage electrophoresis (Shandon Model L24 apparatus) was carried out on Whatman 3MM paper strips in a buffer containing 15 mM ammonium bicarbonate adjusted to pH 8.7 with ammonium hydroxide. High performance liquid chromatography (HPLC) was carried out at ambient temperature on columns (300 x 4 mm) of µ Bondapak C_{18} (Waters Associates) by using Waters Model 510 solvent delivery pumps and a U6K injector. The solvent composition and flow rate (0.8 - 1.5 ml min^{-1}) were programmed with a Waters Model 680 automated gradient controller. Elution profiles were obtained with a Waters Model 441 absorbance detector, operating at 254 or 280 nm, or

by means of an LKB 2140 (photodiode array) Rapid Spectral Detector interfaced to an IBM Personal Computer with a Canon A-1210 printer. Thin layer chromatography (TLC) was performed on precoated (0.2 mm) silica gel 60 F_{254} plastic sheets (Merck). The composition of the solvent systems was as follows: A, 1-butanol-water-acetic acid (5:3:2, by vol.); B, 2-propanol-water-concentrated ammonia (7:2:1, by vol.). Compounds were detected on chromatograms and electrophoretograms by viewing under UV light.

Preparation and properties of the new d(ApA) photoproduct. Aqueous solutions of d(ApA), at a concentration of 0.1 - 0.25 mM, were adjusted to pH 7 with dilute ammonia, and then irradiated with a PCQ-X1 Photochemical Lamp (from Ultra-Violet Products, San Gabriel, California) emitting predominantly at 254 nm. The solution, which was contained in a quartz tube positioned along the central axis of the cylindrical array of circular lamp elements, was stirred by gently bubbling nitrogen through it during irradiation. Ambient temperature (18 - 25^{o}C) was maintained by directing a jet of cool air over the apparatus. Irradiation was continued until the maximum UV absorbance of the solution at 258 nm had decreased by 10% and the minimum absorbance at 227 nm had approximately doubled. The irradiated solution was then lyophilised and the residue was dissolved in a small volume of 15 mM ammonium bicarbonate buffer, pH 8.7, before being subjected to high voltage paper electrophoresis in the same buffer. Under these conditions, the new photoproduct has no net charge and remains close to the origin (to allow for electroendosmotic drift its position is conveniently located by reference to a neutral marker such as 1,3-dimethyluracil). It is thus well separated from unreacted d(ApA) and the Pörschke-type photoproduct whose respective electrophoretic mobilities (as anions) are approximately 2.8 and 5.4 cm kV^{-1} h^{-1}. After excising the relevant band from the electrophoretogram the new photoproduct was extracted from the paper in water and the solution lyophilised. The residue was dissolved in a small volume of water and then eluted with water from a column (25 x 1.5 cm) of Sephadex G-10; 4 ml fractions were collected and those having λ_{max} 282 nm were combined and lyophilised. At this stage, the yield of new photoproduct was ~ 1.8 A_{282} units per mg of irradiated d(ApA). Finally, to remove remaining traces of contaminants, the photoproduct was purified (by multiple injections) on a C_{18} reversed-phase HPLC column which was eluted isocratically with water. At a flow rate of 1.0 ml min^{-1}, the photoproduct gave a single sharp peak with a retention time of 5.3 min. The amorphous

white solid obtained by lyophilisation was also homogeneous on TLC (R_f (A) 0.17; (B) 0.46) and it was used for subsequent spectroscopic characterisation. UV: λ_{max} (pH 7) 282 nm ($\varepsilon \geqslant 5300$ $M^{-1}cm^{-1}$) and 228 ($\geqslant 15800$). Mass spectra: FAB (negative ion) m/z 581, $[M-H]^-$. FAB (positive ion) m/z 583.1780, $[M+H]^+$, calc. for $C_{20}H_{28}N_{10}O_9P$ m/z 583.1779; m/z 289.1272, $[B+3H]^+$, calc. for $C_{10}H_{13}N_{10}O$ m/z 289.1277. ^1H NMR (in D_2O): the low field region of the spectrum is reproduced in Fig.2. It shows signals for two components, in an approximately 3:1 ratio, as follows. Major component: four singlets of equal intensity, assigned to individual protons, at δ 8.06, 8.04, 7.90 and 6.88 plus multiplets for two glycosidic H(1') protons centred at δ 6.19 and 5.94. Minor component : four one proton singlets at δ 8.02, 8.01, 7.65 and 6.84 plus multiplets for two glycosidic protons centred at δ 6.19 (overlapped by signal for major component) and 6.04. At higher field, in the range δ 5.0 - 1.80, there were complex multiplets attributable to the remaining deoxyribose protons of both components.

Acid hydrolysis of the new photoproduct. The HPLC-purified d(ApA) photoproduct (1-5 mg) was dissolved in 1 M HCl (0.5 ml) and heated in a sealed tube at 100°C for 1 h. After evaporation to dryness, the residue was dissolved in 0.05% aqueous trifluoroacetic acid (TFA) and subjected to HPLC on a C_{18} reversed-phase column which was eluted isocratically with 0.05% TFA for 5 min followed by a linear gradient to 0.05% TFA containing 30% methanol after 15 min. The only UV-absorbing component to be eluted in significant quantities (Fig.3) emerged as a sharp peak 3.2 min post-injection (at a flow rate of 1.0 ml min^{-1}). This material, which had λ_{max} 262 nm, was collected and pooled fractions were evaporated to dryness; approximately 8.8 A_{262} units were recovered per mg of hydrolysed photoproduct. The residue was dissolved in water and loaded onto a column (25 x 1.5 cm) of Sephadex G-10 prepared in water. Under these conditions, the UV-absorbing material bound strongly to the Sephadex and the column was washed with several column volumes of water to remove contaminants. On changing the eluent to 0.05% TFA the acid degradation product was immediately eluted and the material thus obtained was homogeneous on HPLC and TLC (R_f (A) 0.25; (B) 0.12). Its UV spectrum (Fig.4) exhibited very distinctive changes between acid, neutral and alkaline pH with apparent spectroscopic pKs of 4.5 and 10.4. Mass spectra: EI m/z 167, M^+; 150, $[M-17]^+$; 125, $[M-42]^+$. FAB (positive ion) m/z 168.0997, $[M+H]^+$, calc. for

$C_5H_{10}N_7$ m/z 168.1000. EI mass spectrum of TMS_4 derivative: m/z 455.2495, M^+, calc. for $C_{17}H_{41}N_7Si_4$ m/z 455.2502.

Subsequently, as described below, this acid degradation product was identified as 4,6-diamino-5-guanidinopyrimidine by detailed comparison of its chromatographic and spectroscopic properties.

Synthesis of 4,6-diamino-5-guanidinopyrimidine (DGPY). A solution of 4,5,6-triaminopyrimidine sulphate (2.5 g) and cyanamide (1.0 g) in water (100 ml) was stirred at 50°C for 80 h. An additional quantity of cyanamide (0.5 g) was then added to the solution which was maintained at 50°C for a further 80 h before being concentrated to half its volume on a rotary evaporator. On standing at room temperature, the solution deposited crystals of 4,6-diamino-5-guanidinopyrimidine sulphate (2.0 g, 67%). The crude product was decolorized with charcoal and crystallised from water or 0.1 M H_2SO_4 to give colourless plates which, on heating, decomposed above 270°C. Found (for a sample dried in vacuo at 50°C for 60 h): C, 22.7; H, 4.1; N, 36.8. Calc. for $C_5H_9N_7.H_2SO_4$: C, 22.6; H, 4.2; N, 37.0 %. UV: λ_{max} (pH 1) 262 nm (ε 10450); (pH 7) 254 (3900); (pH 13) 273 (10300). Mass spectrum (EI) of TMS_4 derivative : m/z 455.2500, M^+, calc. for $C_{17}H_{41}N_7Si_4$ m/z 455.2502. ^1H NMR (in Me_2SO-d_6) : δ 8.27 (bs, 1H, exch. D_2O, C(5)-NH), 7.78 (s, 1H, C(2)H), 7.11 - 6.96 (vb, 4H, exch. D_2O, 2 x guanidinium NH_2), 6.31 (bs, 4H, exch. D_2O, C(4) and C(6) NH_2).

The free base 4,6-diamino-5-guanidinopyrimidine was prepared by stirring an aqueous suspension of equimolar quantities of the finely ground sulphate and barium hydroxide, at ambient temperature, overnight. Barium sulphate was removed by filtration and the free base obtained by evaporation of the filtrate was crystallised from aqueous ethanol as colourless plates m.p. 202-203° decomp. ^{13}C NMR (in Me_2SO-d_6) : δ 107.3 C(5); 151.2, 154.1 and 156.4 (remaining four carbon atoms).

Photoproduct formation in $d(pA)_2$. 2.4 ml of a 0.07 mM solution of $d(pA)_2$, in distilled water adjusted to pH 7 with dilute ammonia, was irradiated at 254 nm (with the PCQ-X1 lamp) in a 1 cm pathlength quartz fluorimeter cell. The UV spectral changes reported by Pörschke[11] were observed and irradiation was continued until the absorbance of the solution at 258 nm had decreased by 12%. After lyophilisation the residue was digested with alkaline phosphatase and examined by reversed-phase HPLC using a linear gradient from 0.05% TFA to 0.05% TFA containing 30% methanol after 18 min. The elution profile showed the pattern (Fig.1) expected for UV-irradiated d(ApA)

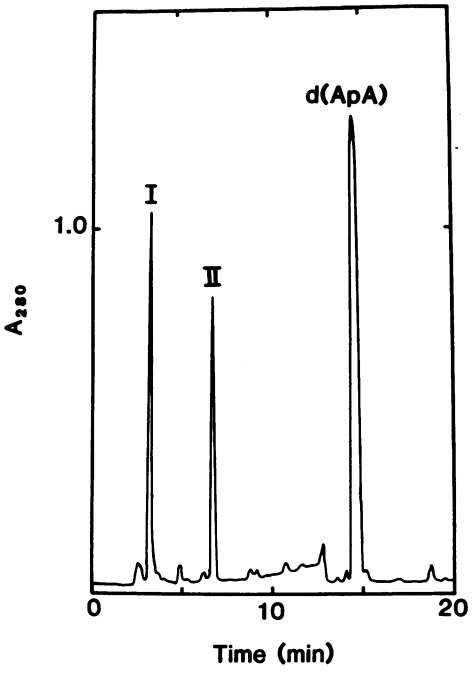


Fig.1. HPLC elution profile (at 280 nm) of d(ApA) irradiated at 254 nm in neutral aqueous solution. Peak I corresponds to the new photoproduct, peak II to the Pörschke-type of photoproduct. The μ Bondapak C_{18} column (300 x 4 mm) was eluted, at a flow rate of 1.5 ml min^{-1}, with a linear gradient from 0.05% aqueous TFA to 0.05% TFA containing 30% methanol after 18 min.

including two major components whose respective retention times and UV spectra (recorded with a photodiode array detector) matched those of the new photoproduct and the species described by Pörschke. To confirm the presence of the new photoproduct, a sample of UV-irradiated $d(pA)_2$ was hydrolysed with 1 M HCl at 100°C for 1 h. The acid hydrolysate was shown, by HPLC with diode array detection, to contain material having the same retention time and UV spectrum as for DGPY.

Photoproduct formation in poly(dA) and poly(rA). A solution (3.2 ml) containing 7 A_{260} units of poly(dA) in 0.10 M Na^+, pH 7.0 buffer (0.05 M NaCl + 0.025 M Na_2HPO_4 adjusted to pH 7 with HCl) was saturated with nitrogen and irradiated at 254 nm in a 1 cm pathlength quartz fluorimeter cell. The characteristic absorption changes reported by Pörschke were noted and irradiation was continued until the absorbance at 227 nm had doubled. The irradiated solution was then dialysed extensively against distilled

water to remove buffer salts, lyophilised, treated with 1 M HCl at 100°C for 1 h, and evaporated to dryness. On reversed-phase HPLC with a 0.05% TFA - methanol gradient the acid hydrolysate gave a prominent peak corresponding to DGPY. This material was collected and its identity as DGPY was verified from the pH dependence of its UV absorption and by TLC.

Similar results were obtained when poly(dA) was irradiated in distilled water at neutral pH. However, when poly(rA) was irradiated under these conditions, only minimal changes were observed in its UV spectrum and no trace of DGPY could be detected by HPLC in the HCl hydrolysate of the irradiated polyribonucleotide.

RESULTS

Isolation and spectroscopic characterisation

In preliminary analytical experiments, the time course of the photoreaction in d(ApA) irradiated at 254 nm in neutral aqueous solution was monitored by reversed-phase HPLC using a 0.05% TFA - methanol gradient. As illustrated in Fig.1, this clearly revealed the independent formation of two major primary photoproducts. To avoid possible breakdown of the photoproducts in the acidic solvent employed for HPLC, they were separated

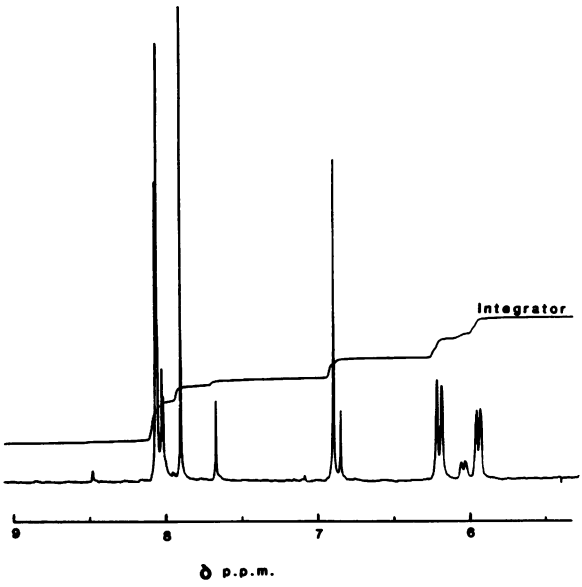


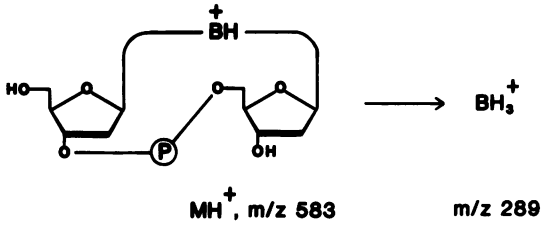
Fig.2. Low field region of the ^1H NMR spectrum of the new d(ApA) photoproduct in D_2O. The photoproduct is postulated to be a mixture of two diastereomers with the structure (4) shown in Scheme 3.

on a preparative scale by high voltage paper electrophoresis at pH 8.7. Under these conditions, one photoproduct behaved as if it had no net charge and remained close to the origin while the other migrated as an anion with considerably higher mobility than unreacted d(ApA). From its UV absorption spectrum (λ_{max} 263 nm, λ_{min} 234 nm) and its anionic character the latter photoproduct (with the longer retention time on HPLC) could be identified as the same type of species as previously described by Pörschke[11]. There was, however, no precedent for the electrophoretically neutral photoproduct, with absorption maxima at 228 and 282 nm, which was eluted first on HPLC. Both photoproducts were reasonably stable to further irradiation at 254 nm in solution and there was no evidence for their photochemical interconversion.

After extraction of the new d(ApA) photoproduct from paper electrophoretograms with water, it was further purified by chromatography on Sephadex G-10 followed by elution from a reversed-phase HPLC column with water. Thus obtained, the photoproduct behaved as a homogeneous substance on HPLC and TLC. Nonetheless, its ^1H NMR spectrum indicated that it comprised a mixture of two components with similar structures in an approximately 3 : 1 ratio. This is evident from the low field ^1H NMR spectrum, shown in Fig.2, where each component gives rise to a similar pattern of four singlet resonances in the aromatic region as well as two glycosidic H(1') signals.

The molecular mass of the photoproduct was determined as 582 by FAB mass spectrometry, the positive ion spectrum showing the protonated molecule, $[M+H]^+$, at m/z 583, while the negative ion spectrum displayed the $[M-H]^-$ species at m/z 581. High resolution mass measurements established the photoproduct's composition as $C_{20}H_{27}N_{10}O_9P$ which corresponds to that of d(ApA) + H_2O. In addition, the photoproduct was shown to contain nine active hydrogens, as compared to seven for d(ApA), by using a deuterated matrix to induce total deuterium exchange of active hydrogens during FAB mass spectrometry.

Tandem mass spectrometry with collisional activation[18] was then employed to identify daughter ions produced by fragmentation of the protonated molecule, $[M+H]^+$. The most abundant daughter ion occurred at m/z 289. On re-examination, the conventional (non-tandem) positive ion FAB spectrum also showed this ion (at low relative abundance) and its composition, $C_{10}H_{13}N_{10}O$, provided compelling evidence that the photoproduct is formed from d(ApA) by covalent linking of the two adenine bases accompanied by incorporation of the elements of one molecule of water. As



Scheme 1. Fragmentation pathway whereby the daughter ion with m/z 289 is derived from a protonated molecule of the d(ApA) photoproduct.

depicted in Scheme 1, the observed fragmentation of $[M+H]^+$ to give m/z 289 thus involves cleavage of both glycosidic bonds and loss of two deoxyribose moieties in the photoproduct by a mechanism similar to that which generates abundant $[B+H]^+$ ions in the mass spectra of common purine nucleosides[19]. Ions analogous to m/z 289 have also been reported in the mass spectra of synthetic base-base cross-linked ribonucleosides[20].

Degradation to 4,6-diamino-5-guanidinopyrimidine

The internucleotide bond of the new photoproduct was resistant to cleavage by S_1 nuclease and the phosphodiesterase enzymes from snake venom and bovine spleen. To facilitate structure elucidation we therefore attempted to liberate the heterocyclic base moiety from the deoxyribose-phosphate backbone by the action of dilute acid. When the photoproduct was exposed to 1 M HCl at 95°C its UV absorption spectrum exhibited a series of changes before a constant spectrum with λ_{max} 262 nm was attained after 30

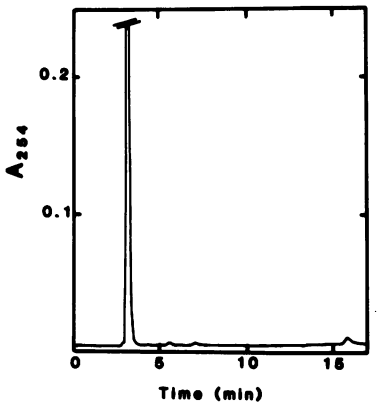


Fig.3. HPLC elution profile (at 254 nm) of acid hydrolysate derived from the new d(ApA) photoproduct. For elution conditions see text.

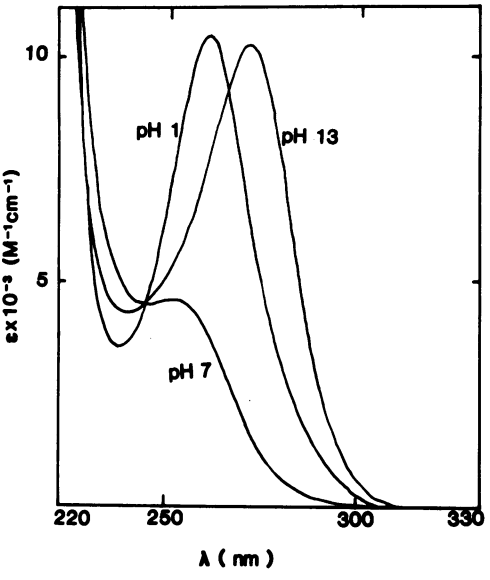
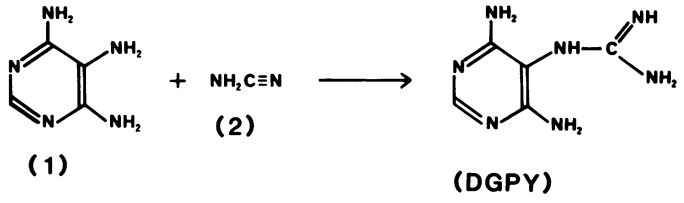


Fig.4. UV spectra of 4,6-diamino-5-guanidinopyrimidine at acid, neutral and basic pH.

min. Samples were thereafter treated with 1 M HCl at 100°C for 1 h. As illustrated in Fig.3, HPLC analysis of the acid hydrolysate showed only one significant UV-absorbing component with a very short retention time. This material was collected and was conveniently purified by virtue of its strongaffinity for Sephadex G-10 at neutral pH; after extensive washing with water it was eluted quantitatively with 0.05% TFA. The acid degradation product thus obtained was chromatographically homogeneous and, as shown in Fig.4, exhibited most unusual changes in its UV absorption with increasing pH. In particular, between pH 1 and pH 7 (pK ~ 4.5) there is an approximately 3-fold decrease in extinction coefficient accompanying a blue-shift of the absorption maximum. This behaviour resembles that reported for 4,6-diamino-5-formylaminopyrimidine by Cavalieri and Bendich[21].

The positive ion FAB mass spectrum of the acid degradation product showed an intense peak at m/z 168 which was attributed to $[M+H]^+$. At high resolution, however, the peak appeared as a closely spaced doublet. Accurate mass measurements on the component with higher m/z ratio indicated a composition of $C_5H_9N_7$ for the parent molecule. This was confirmed by analysis of the EI mass spectrum of its tetrakistrimethylsilyl derivative. An EI mass spectrum of the underivatized hydrolysis product showed in



Scheme 2. Synthesis of 4,6-diamino-5-guanidinopyrimidine (DGPY).

addition to M^+ at m/z 167, abundant ions at m/z 150 and 125. Prominent [M-17] and [M-42] ions are a feature of the EI mass spectra of aminoguanidinopyrimidines[16].

On the basis of the above data, the acid degradation product was tentatively identified as 4,6-diamino-5-guanidinopyrimidine. Comparison by TLC with synthetic samples of 4,5-diamino-2-guanidinopyrimidine, 4,6-diamino-2-guanidinopyrimidine and 4,5-diamino-6-guanidinopyrimidine[16] allowed these alternative structural isomers to be excluded. Noting that formylation of 4,5,6-triaminopyrimidine with formic acid occurs specifically at the C(5)-amino group[22], we synthesised 4,6-diamino-5-guanidino-pyrimidine (DGPY) by prolonged reaction of 4,5,6-triaminopyrimidine sulphate (1) with cyanamide (2) in aqueous solution at 50°C (Scheme 2). This afforded DGPY, as its crystalline sulphate, in good yield. Its elemental composition was established by accurate mass measurements on the molecular ion of its tetrakistrimethylsilyl derivative and the presence of a guanidino function in DGPY was confirmed by its production of a magenta colour with diacetyl/α-naphthol spray reagent[23]. The position of substitution followed from the observation of only four signals in the ^{13}C NMR spectrum of the free base since the C(4) and C(6) carbon atoms are equivalent. Furthermore, monosubstitution of 4,5,6-triaminopyrimidine can give only two structural isomers and the reaction product was distinguished by TLC and its UV absorption from an authentic sample[16] of 4,5-diamino-6-guanidino-pyrimidine.

The identity of the acid degradation product derived from the new d(ApA) photoproduct as DGPY was demonstrated by detailed spectroscopic and chromatographic comparison. The two substances co-chromatographed on HPLC and TLC and their UV spectra at pH 1, pH 7 and pH 13 were coincident. In addition, the EI mass spectra of their respective tetrakistrimethylsilyl derivatives gave almost identical fragmentation patterns.

No DGPY was detected when the Pörschke-type of photoproduct from d(ApA) was subjected to similar acid treatment.

Dinucleotide and polynucleotide photoproducts

The specific production of DGPY on acid hydrolysis was used to assess whether formation of the new photoproduct occurs at the dinucleotide or the polynucleotide level. When irradiated at 254 nm in neutral aqueous solution the dinucleotide $d(pA)_2$ and poly(dA) showed the same qualitative changes in their UV absorption as reported earlier by Pörschke[10,11]. Acid hydrolysates prepared from the irradiated samples were examined by HPLC and both were found to contain substantial amounts of DGPY. Further evidence for formation of the photoproduct in $d(pA)_2$ was obtained by treating the irradiated dinucleotide with alkaline phosphatase. On HPLC, the enzyme digest was shown to contain a species whose retention time and UV spectrum corresponded to those expected for the new d(ApA) photoproduct.

In marked contrast to poly(dA), its ribonucleotide analogue poly(rA) is very resistant to photochemical change[10]. As judged by the absence of DGPY from acid hydrolysates of UV-irradiated poly(rA), formation of the new photoproduct does not occur in this homopolymer.

DISCUSSION

Photoproduct Formation

Hitherto, the photoreactivity observed in UV-irradiated oligomers and polymers of deoxyadenylic acid has been attributed to specific formation of the type of photoproduct described by Pörschke. However, it is clear from the results presented here that the concurrent formation of a second major photoproduct must also be taken into account. The two photoproducts from d(ApA) are readily distinguished by their UV absorption and electrophoretic mobility. At neutral pH the Pörschke-type of photoproduct migrates as an anion but the new photoproduct behaves as an internal salt in having no net charge. This difference has been exploited for their preparative separation (and the recovery of unchanged d(ApA)) by high voltage paper electrophoresis.

Pörschke noted [11] that the conversion of $d(pA)_2$ into the photoproduct which he isolated was not quantitative. To some extent this can be explained by our demonstration that comparable amounts of a second photoproduct are produced in UV-irradiated $d(pA)_2$. Nonetheless, our experience with d(ApA) suggests that both types of photoproduct are liable to photodegradation upon prolonged irradiation in solution since their

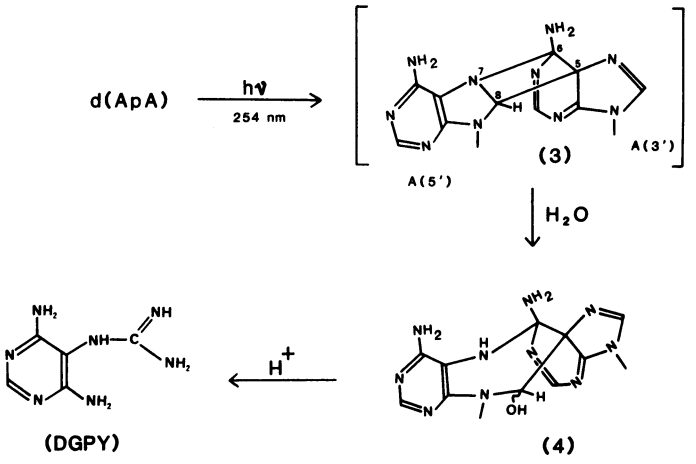
respective yields tend to plateau with increasing UV fluence. It is possible that the two photoproducts are derived, by competing reaction pathways, from a common singlet excited state precursor since the course of photolysis of d(ApA) was essentially the same for aerated solutions and those saturated with nitrogen. In support of a singlet mechanism, the new photoproduct was not formed when d(ApA) was irradiated at wavelengths >290 nm in the presence of 1 M acetone as triplet photosensitiser. We have also observed that it is not formed in significant quantity when d(ApA) is irradiated at 254 nm in frozen aqueous solution.

Structural Considerations

The results from high resolution mass spectrometry indicate that the new d(ApA) photoproduct is an intramolecular photoadduct which arises by covalent linking of the two adenine bases with concomitant incorporation of the elements of one molecule of water. However, the purified photoproduct is shown by ^{1}H NMR to be a mixture of two discrete components which exhibit completely analogous signal patterns in the low field region of the spectrum (Fig.2). Since the two components also have extremely similar physical properties, and have resisted many attempts to resolve them by HPLC, TLC and paper chromatography, we conclude that they are stereoisomers with closely related structures. In this context, it is also pertinent to note that acid treatment of the photoproduct gives only a single UV-absorbing degradation product, namely DGPY.

The structure of DGPY makes it reasonably certain that the new photoproduct is formed by a photoaddition reaction involving the N(7) - C(8)bond of one of the adenine bases in d(ApA). Moreover, it is likely that N(7) becomes linked to a carbon atom in the other adenine base which is flanked by two nitrogen atoms so that a guanidino function can be generated on acid hydrolysis. A further constraint on possible structural models for the photoproduct concerns the stereochemical feasibility of the photoaddition process. It is known[24-26] from NMR, and other spectroscopic measurements, that d(ApA) in aqueous solution adopts a (time-averaged) stacked right-handed helical conformation with the bases disposed approximately as in double stranded DNA. The photoreaction might therefore be expected to involve reactive centres that are juxtaposed in this stacked conformation with the correct orientation for product formation.

In light of the above considerations, we propose the sequence outlined in Scheme 3 to account for the formation of the new d(ApA) photoproduct and its subsequent degradation by acid to DGPY. It must be emphasised that the



Scheme 3. Proposed mechanism of formation of the new d(ApA) photoproduct (4) which gives DGPY on acid hydrolysis. For clarity, the deoxyribose-phosphodiester backbone has been omitted.

structure (4) assigned to the photoproduct is not regarded as definitive but it is generally consistent with the observed spectroscopic and physical properties. The primary photochemical event is postulated to be a cycloaddition reaction yielding an azetidine species (3) in which the N(7) and C(8) atoms of the 5'-adenine in d(ApA) are linked respectively to the C(6) and C(5) positions of the 3'-adenine. This mode of photoaddition is compatible with the geometry of a stacked d(ApA) molecule and is precisely analogous to the mechanism we have previously proposed[27] to account for the formation of a thymine-adenine photoadduct in UV-irradiated d(TpA) and DNA. The highly strained (and protonated) azetidine ring of the primary photoadduct (3) is assumed to undergo rapid hydrolytic cleavage to yield the isolated photoproduct (4). This sequence of photoaddition to give an azetidine followed by fission of the four-membered ring bears some resemblance to the currently accepted mechanism[28,29] for the production of bipyrimidine (6-4) photoadducts derived from cytosine. Finally, on treatment with acid, the heterocyclic base moiety of the photoproduct (4) is fragmented so that the aromatic pyrimidine ring forms the nucleus of the only UV-absorbing stable hydrolysis product, DGPY.

The structure assigned to the photoproduct (4) is fully in accord with its elemental composition and that of the $[B+3H]^+$ fragment ion (Scheme 1) determined by mass spectrometry. Incorporation of a molecule of water by

the proposed route should generate a mixture of two diastereomers giving rise to the observed pattern of signals in the low field ^{1}H NMR spectrum (Fig.2). Assignment of the signals to specific protons has not been attempted particularly as their chemical shifts may be influenced by anisotropic effects in such a molecule. The UV spectrum of the new photoproduct closely matches that reported[30] for a stable azetidine species which is formed by photocycloaddition between the N(7) - C(8) double bond of adenine and the C(5) - C(6) double bond of thymine when these bases are linked together by a trimethylene bridge between their respective N(9) and N(1) positions. Both molecules possess a similar substituted triamino-pyrimidine fragment as their major chromophore. As the photoproduct is electrophoretically neutral at pH 8.7, one of the nitrogen atoms in the heterocyclic base moiety must be protonated and thus balance the negative charge associated with the phosphodiester backbone.

Detection in Polynucleotides

The highly specific conversion of the photoproduct into DGPY on acid hydrolysis provides a useful method for its detection in UV-irradiated oligo- and poly-nucleotides. The conversion yield, calculated from a minimal estimate of the molar extinction coefficient of the photoproduct, is $\geq$ 50%. Any DGPY present in acid hydrolysates of UV-irradiated samples can be rapidly isolated by HPLC and then quantified by spectrophotometry. By this means, we have demonstrated formation of the new photoproduct in $d(pA)_2$ and poly(dA). It should be possible to amplify the sensitivity of the assay very considerably in cases where a polynucleotide contains radioactive adenine bases with an appropriate labelling pattern.

Conclusions

The findings discussed in this paper provide a striking example of how structural and conformational factors can radically influence the photoreactivity of polynucleotides and their components. Thus, under irradiation conditions where monomeric adenine derivatives are essentially stable, the dimer d(ApA) undergoes two quite distinct intramolecular photoreactions. Their combined quantum yield is presumably close to the value of 1×10^{-3} determined by Pörschke[13] for the photodegradation of d(ApA) by 248 nm radiation. From preliminary measurements, where the absorbed UV dose was determined by actinometry with 1,3-dimethyluracil[31], we estimate the quantum yield for formation of the new photoproduct in d(ApA) to be $\geq 3 \times 10^{-4}$. A value twice as large is predicted[11], on statistical

grounds, for its formation in poly(dA). Remarkably, there is no detectable formation of the new photoproduct in UV-irradiated poly(rA). The same situation pertains to the photoproduct characterised by Pörschke so both photoreactions appear to be specific for dimer units having a DNA-type of internucleotide linkage. As discussed by Pörschke[13], this most probably reflects known differences between the stacking geometry of single stranded deoxyriboadenylate chains and that of their RNA counterparts.

Whether or not the new d(ApA) photoproduct contributes, as a minor lesion, to biologically significant photodamage in native DNA remains to be established. Its formation in poly(dA) suggests that similar photoaddition should be feasible between adjacent adenine bases in single stranded DNA. The more rigid conformation of double stranded DNA may, however, place some constraints on the photoreaction. As we have proposed stereochemically analogous photoaddition processes to account for the formation of this dimeric adenine photoproduct (Scheme 3) and of the thymine-adenine photoadduct in TA doublets[27], it is pertinent to note that the quantum yield of the latter reaction is five-fold lower in double stranded than in single stranded DNA[7]. Pörschke has pointed out[11] that the formation of a dimeric adenine photoproduct opposite to a thymine dimer in UV-irradiated DNA would cause a potentially very harmful lesion that could not be repaired by template-directed repair enzymes. Although such an event would have a very low probability, the local distortion produced in the DNA helix by a pre-existing thymine dimer[32] might facilitate photoreaction between the adjacent adenine bases on the complementary strand.

ACKNOWLEDGEMENTS

This work was supported by the Science and Engineering Research Council and, in part, by grant GM 29812 from the National Institute of General Medical Sciences. The authors are very grateful to VG Analytical, Manchester, for measurement of tandem mass spectra, and to Dr D.L. Ladd of Smith Kline & French Laboratories, Philadelphia, for providing samples of three diaminoguanidinopyrimidines.

* Author to whom correspondence should be addressed.

REFERENCES

1. Patrick, M.H. and Rahn, R.O. (1976) in Photochemistry and Photobiology of Nucleic Acids, Wang, S.Y. Ed., Vol. II, pp. 35-95, Academic Press, London.

2. Harm, W. (1980) Biological Effects of Ultraviolet Radiation, Cambridge University Press, Cambridge.
3. Cadet, J., Voituriez, L., Grand, A., Hruska, F.E., Vigny, P. and Kan, L.-S. (1985) Biochimie 67, 277-292.
4. Elad, D. (1976) in Photochemistry and Photobiology of Nucleic Acids, Wang, S.Y. Ed., Vol I, pp. 357-380, Academic Press, London.
5. Livneh (Noy), E., Tel-Or, S., Sperling, J. and Elad, D. (1982) Biochemistry, 21, 3698-3703.
6. Bose, S.N., Davies, R.J.H., Sethi, S.K. and McCloskey, J.A. (1983) Science 220, 723-725.
7. Bose, S.N. and Davies, R.J.H. (1984) Nucleic Acids Res. 12, 7903-7914.
8. Becker, M.M. and Wang, J.C. (1984) Nature, 309, 682-687.
9. Gallagher, P.E. and Duker, N.J. (1986) Mol. Cell. Biol. 6, 707-709.
10. Pörschke, D. (1973) Proc. Nat. Acad. Sci. USA, 70, 2683-2686.
11. Pörschke, D. (1973) J. Am. Chem. Soc. 95, 8440-8446.
12. Ivanchenko, V.A., Titschenko, A.I., Budowsky, E.I., Simukova, N.A. and Wulfson, N.S. (1975) Nucleic Acids Res. 2, 1365-1373.
13. Pörschke, D. (1975) Nucleic Acids Res. 2, 1621-1627.
14. Gasparro, F.P. and Fresco, J.R. (1986) Nucleic Acids Res. 14, 4239-4251.
15. Joshi, P.C. and Davies, R.J.H. (1981) J. Chem. Res.(S) 227; J. Chem. Res.(M) 2701-2732.
16. Ladd, D.L. (1982) J. Heterocyclic Chem. 19, 917-921.
17. Sethi, S.K., Smith, D.L. and McCloskey, J.A. (1983) Biochem. Biophys. Res. Commun. 112, 126-131.
18. Crow, F.W., Tomer, K.B., Gross, M.L., McCloskey, J.A. and Bergstrom, D.E. (1984) Anal. Biochem. 139, 243-262.
19. McCloskey, J.A. (1974) in Basic Principles in Nucleic Acid Chemistry, Ts'o, P.O.P. Ed., Vol I, pp. 209-309, Academic Press, New York.
20. Sethi, S.K., Gelijkens, C.F., McCloskey, J.A., Shapiro, R. and Brandes, B.A. (1983) Biomed. Mass Spectrom. 10, 665-670.
21. Cavalieri, L.F. and Bendich, A. (1950) J. Am. Chem. Soc. 72, 2587-2593.
22. Cavalieri, L.F., Tinker, J.F. and Bendich, A. (1949) J. Am. Chem. Soc. 71, 533-536.
23. Foster, M.C. and Ashton, G.C. (1953) Nature 172, 958-959.
24. Kondo, N.S., Fang, K.N., Miller, P.S. and Ts'o, P.O.P. (1972) Biochemistry 11, 1991-2003.
25. Cheng, D.M. and Sarma, R.H. (1977) J. Am. Chem. Soc. 99, 7333-7348.
26. Olsthoorn, C.S.M., Bostelar, L.J., van Boom, J.H. and Altona, C. (1980) Eur. J. Biochem. 112, 95-110.
27. Bose, S.N., Kumar, S., Davies, R.J.H., Sethi, S.K. and McCloskey, J.A. (1984) Nucleic Acids Res. 12, 7929-7947.
28. Wang, S.Y. (1976) in Photochemistry and Photobiology of Nucleic Acids, Wang, S.Y. Ed., Vol. I, pp. 295-356, Academic Press, London.
29. Franklin, W.A., Doetsch, P.W. and Haseltine, W.A. (1985) Nucleic Acids Res. 13, 5317-5325.
30. Wenska G., Paszyc, S. and Skalski, B. (1976) Bull. Acad. Polon. Sci. Ser. Sci. Chim. 24, 517-526.
31. Rahn, R.O. and Sellin, H.G. (1979) Photochem. Photobiol. 30, 317-318.
32. Pearlman, D.A., Holbrook, S.R., Pirkle, D.H. and Kim, S-H. (1985) Science 227, 1304-1308.