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The photochemistry of d(T-A) in aqueous solution and in ice

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ABSTRACT

When d(T-A) is irradiated at 254 nm in aqueous solution an internal photoadduct is formed between its constituent adenine and thymine bases. The resultant photoproduct, designated TA*, arises from a singlet excited state precursor; a similar photoreaction is not observed with d(C-A) or d(T-G). In contradistinction, irradiation of d(T-A) in frozen aqueous solution yields a dimeric photoproduct in which two d(T-A) molecules are coupled together by a (6-4) photoadduct linkage between their respective thymine bases. Both photoproducts have been extensively characterised by a combination of electron impact and fast atom bombardment mass spectrometry, UV, CD, ^{1}H NMR and fluorescence spectroscopy. Acid treatment of TA* gives 6-methylimidazo[4,5-*b*]pyridin-5-one whose identity was established by an independent chemical synthesis involving photorearrangement of 6-methylimidazo[4,5-*b*]pyridine *N*(4)-oxide. A tentative mechanism is presented to account for the acid degradation of TA*. The structure of the dimeric ice photoproduct follows from its cleavage, by snake venom phosphodiesterase, to 5'-dAMP and the (6-4) bimolecular photoadduct of thymidine; on acid hydrolysis it gives adenine and 6-(5'-methyl-2'-oxopyrimidin-4'-yl)thymine.

INTRODUCTION

The mutagenic and lethal effects of short wavelength ultraviolet (UV) radiation on biological systems arise primarily from photochemical alterations to the structure of DNA[1,2]. It is now well established[3] that a number of photoreactions involving the pyrimidine bases, thymine and cytosine, can occur in DNA, though the distribution and relative yields of the photoproducts depend markedly on its conformation and base composition. Characterisation of the major pyrimidine photoproducts, such as cyclobutyl photodimers and bipyrimidine (6-4) photoadducts, has been considerably facilitated because (i) they are sufficiently stable to be isolated, without substantial structural modification, from acid hydrolysates of DNA, and (ii) the photoreactions leading to their formation can be readily reproduced, at the monomer level, by irradiating the relevant pyrimidine bases, nucleosides or mononucleotides in aqueous solution or in an ice matrix[4,5].

By contrast, monomeric purine bases and their derivatives are extremely resistant to photochemical change[3,6]. Their lack of reactivity, coupled with the failure[1,7] to isolate any photoproducts derived from adenine or guanine from hydrolysates of UV-irradiated DNA, has led to the prevalent assumption that the purine bases in DNA are virtually unaffected by UV radiation and do not, therefore, constitute significant targets for photobiological damage. There is, however, convincing evidence that the photoreactivity of a purine base can be greatly enhanced when it is incorporated into a polynucleotide structure. Thus, although 5'-dAMP in neutral aqueous solution is quite stable towards short wavelength radiation, the adenine bases of d(pA-A) and higher oligodeoxyadenylates readily form a dimeric photoproduct (of unknown structure) when irradiated under the same conditions[8]. These findings led us to select deoxydinucleoside monophosphates as model systems for evaluating the photoreactivity of the purine bases in DNA. We reasoned that the physical proximity of the two heterocyclic bases in these molecules might permit pathways for energy transfer and photochemical reaction that are not accessible to monomeric purine derivatives in solution. In the course of examining the purine-containing deoxydinucleoside monophosphate sequences that occur as nearest neighbour doublets in DNA, we discovered that d(T-A) undergoes two quite distinct photoreactions when irradiated, at 254 nm, in aqueous solution and in ice. On irradiation in aqueous solution, the adenine and thymine bases of d(T-A) become covalently linked to form an intramolecular photoadduct[9], whereas irradiation in frozen aqueous solution gives a photoproduct in which two d(T-A) molecules are coupled together through their respective thymine bases[10].

This paper gives details of the preparation, properties, and characterisation of the two d(T-A) photoproducts. Acid hydrolysis of the solution photoproduct, which we have designated TA*, converts it into a strongly fluorescent modified base whose identity as 6-methylimidazo[4,5-*b*]pyridin-5-one has been confirmed by chemical synthesis. Elsewhere[9,11], definitive evidence is presented for formation of TA* in simple oligodeoxyribonucleotides, the alternating copolymer poly(dA-dT), and both native and denatured DNA.

EXPERIMENTAL

Materials

Deoxydinucleoside monophosphates. The sodium salts of d(T-A) and d(A-T), and the ammonium salts of d(T-G) and d(T-T) were obtained from Sigma; the

ammonium salt of d(C-A) was from Collaborative Research.

Other Chemicals. The following were purchased from the commercial suppliers indicated: thymidine and the sodium salt of 5'-dAMP (Sigma), thymine and caffeine (BDH), adenine (Fluka), imidazo[4,5-*b*]pyridine and 2-amino-5-methylpyridine (Aldrich), *N,O*-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (Pierce), *N,O*-bis(trimethylsilyl-d_9)acetamide and trimethylchlorosilane-d_9 (Merck Isotopes), Sephadex G-10 (Pharmacia).

Phosphodiesterases. The enzyme from snake venom (*Crotalus durissus*) was obtained from Boehringer Mannheim, and the bovine spleen enzyme from Sigma.

Methods

UV spectra were recorded on a Cary 118 spectrophotometer, IR spectra on a Perkin-Elmer Model 257 grating infrared spectrometer, fluorescence spectra on a Perkin-Elmer MPF-44B spectrofluorimeter with a DCSU-2 differential corrected spectra attachment, and CD spectra on a Cary 61 instrument. Unless otherwise indicated, ^{1}H NMR spectra were obtained with a Bruker WM 250 Fourier transform spectrometer; tetramethylsilane was used as internal standard for spectra run in Me_2SO-d_6 but spectra run in D_2O were calibrated only by reference to the DHO signal assumed to have δ 4.80. The ^{13}C NMR of compound (6) was obtained by courtesy of the SERC Very High Field NMR Service at the University of Sheffield.

Electron ionization mass spectra were recorded using a MAT 731 or AEI MS-902 mass spectrometer operating at 70 eV ionising energy; exact mass values were determined by peak matching, at resolution 12,000, using perfluoroalkanes as internal standard. Trimethylsilyl (TMS) derivatives for mass spectrometry were prepared by heating 1-10 µg of sample in *N,O*-bis(trimethylsilyl)trifluoroacetamide, trimethylchlorosilane and dimethylformamide (80:1:20) at 100°C for 1 h. Perdeuterotrimethylsilyl derivatives were similarly prepared using *N,O*-bis(trimethylsilyl-d_9)acetamide and trimethylchlorosilane-d_9. Fast atom bombardment (FAB) mass spectra were obtained from the Middle Atlantic Mass Spectrometry Laboratory, Johns Hopkins University, Baltimore using a Kratos MS-50 instrument and thioglycerol matrix.

Thin layer chromatography (TLC) was performed on precoated (0.2 mm) silica gel 60 F_{254} plastic sheets (E. Merck, Darmstadt) and paper chromatography on Whatman 3MM strips. The following solvent systems were used: A, 1-propanol/water, 7:3; B, 1-butanol/water/acetic acid, 80:30:12; C, 1-butanol/water/acetic acid, 5:3:2; D, isobutyric acid/water/concentrated ammonia, 66:33:1; E, ethyl acetate/water/1-propanol, 4:2:1 (upper phase). High voltage electrophoresis was carried out on Whatman 3MM paper strips

using a Shandon apparatus (Model L24). The pH 2.5 and 3.4 buffers contained 15 mM ammonium formate adjusted to the appropriate pH with formic acid, the pH 9.0 buffer 20 mM ammonium bicarbonate adjusted to pH 9.0 with dilute ammonia. Quoted values of (apparent) electrophoretic mobility have been measured relative to caffeine as zero to correct for electroendosmotic effects. Compounds were detected on chromatograms and electrophoretograms by viewing under UV light.

Digestion with snake venom phosphodiesterase was carried out at 37°C in ∿ 30 mM sodium glycinate buffer, pH 8.8; the digestion products were analysed by high voltage paper electrophoresis in 20 mM sodium glycinate buffer, pH 8.8, or at pH 9.0. With bovine spleen phosphodiesterase, samples were digested at 37°C in ∿ 50 mM sodium succinate buffer, pH 6.5; the products were analysed by electrophoresis in 20 mM sodium succinate buffer, pH 6.5, or at pH 9.0.

Preparation and properties of the d(T-A) solution photoproduct, TA*.

A 0.1 mM aqueous solution of d(T-A) was adjusted to pH 7 with dilute ammonia and irradiated, under nitrogen, with a PCQ-X1 Photochemical Lamp (Ultra-Violet Products, San Gabriel, California) emitting predominantly at 254 nm. (It is essential to exclude 185 nm radiation). The solution was contained in a quartz tube positioned along the central axis of the cylindrical array of UV lamps. The course of the photoreaction was monitored by recording the UV spectrum of the d(T-A) solution at regular intervals and irradiation was continued until the absorbance at 260 nm fell to half its initial value. The irradiated solution was then lyophilised and the residue subjected to preparative paper chromatography in solvent A. This separates TA* (R_f = 0.26) from unchanged d(T-A) (R_f = 0.38). The TA* was extracted from the chromatogram with water, lyophilised, and finally purified by chromatography on a column (20 x 1.7 cm) of Sephadex G-10. The column was eluted with water and fractions shown by their UV spectra to contain TA* were pooled and lyophilised. The photoproduct, TA*, was thus obtained as a white amorphous solid in an overall yeild of 30%; it was homogeneous on TLC (R_f (A) 0.36, (C) 0.20, (D) 0.29) and electrophoresis.

The UV and CD spectra of TA*, at pH 7, are reproduced in reference 9. Its ^1H NMR spectrum recorded in D_2O, with tetramethylammonium chloride (δ 3.19) as internal standard, showed the following salient features: δ 7.85 (s, 1H, base C-H), 7.29 (s, 1H, base C-H), 6.41 (m, 1H, deoxyribose C(1')H), 6.01 (m, 1H, deoxyribose C(1')H), 5.03 (s, 1H, base C-H), 1.58 (s, 3H, T-derived CH_3); in addition several complex multiplets, attributable to deoxyribose ring protons, were observed in the region δ 4.3-1.8. The mass

spectrum of TA* is discussed in the main text.

Acid hydrolysis of TA*. A solution of TA* (1 mg) in 1 M HCl (1 ml) was heated at 100°C for 4 h in a sealed glass tube. The hydrolysate was then evaporated to dryness on a rotary evaporator; to completely remove HCl, the residue was repeatedly dissolved in water and re-evaporated. Analysis of the hydrolysate by TLC showed a single fluorescent species and no trace of thymine, adenine or hypoxanthine. The fluorescent hydrolysis product was isolated by high voltage electrophoresis at pH 2.5 (mobility 3.3 cm kV^{-1} h^{-1}). After extraction from the paper with water, and removal of ammonium formate by repeated lyophilisation, it was applied to a column of Sephadex G-10 (20 x 1.7 cm) in water. The fluorescent product was eluted from the column with water and, thus obtained, it was homogeneous on TLC, paper chromatography and electrophoresis. Its elemental composition was established as $C_7H_7N_3O$ by high resolution mass spectrometry (found, and calculated for $C_7H_7N_3O$: *m/z* 149.0589); the presence of two active hydrogens was indicated by the incorporation of two TMS groups (M^+, *m/z* 293) on trimethylsilylation. Ultimately, the fluorescent hydrolysis product was identified as 6-methylimidazo[4,5-*b*]pyridin-5-one (6) by detailed spectroscopic (UV,IR, fluorescence and ^1H NMR) and chromatographic comparison with synthetic material (see below).

Preparation and properties of the d(T-A) ice photoproduct (13). 100 ml batches of a 0.1 mM solution of d(T-A) in water were frozen in trays at -20°C (in a deep freeze room) to give a uniform layer 5 mm thick. The frozen solution was placed 10 cm below two 8 W germicidal strip lamps (Sylvania G8T5, emitting predominantly at 254 nm) and irradiated for 1 h after which the solution was thawed, refrozen, and irradiated for a further period of 1 h. The total incident fluence (measured with a J-225 short wave UV meter from Ultra-Violet Products, San Gabriel, California) was ∿70 kJ m^{-2}. Under these conditions, ∿40% of the original d(T-A) was converted into the ice photoproduct. The photoproduct was isolated by preparative high voltage paper electrophoresis, at pH 3.4, of the residue obtained on evaporation of the thawed irradiated solution to dryness. This separated the photoproduct (mobility 1.6 cm $kV^{-1}h^{-1}$) from unchanged d(T-A) (mobility 1.4 cm $kV^{-1}h^{-1}$). The photoproduct was extracted from the paper with water and lyophilised repeatedly to remove all traces of ammonium formate. It was finally purified by elution with water from a column of Sephadex G-10 (20 x 1.7 cm) and obtained as a white amorphous solid which was homogeneous on electrophoresis and TLC (R_f (A) 0.39, (C) 0.07, (D) 0.40). UV:λ_{max} (pH 7) 259 nm (ε 20300) and 319 (4400). Corrected

fluorescence (pH 7): λ_{max} (excitation) 321, λ_{max} (emission) 388 nm. ^1H NMR (Me_2SO-d_6) δ 10.31 (bs, 1H, exch. D_2O, dihydrothymine H(3)), 8.43 (2 overlapping singlets, 2H, 2 x H(2) of dA), 8.03 (s, 1H, pyrimidinone H(6)) 7.28 (bs, 4H, exch. D_2O, 2 x NH_2 of dA), 6.35 (m, 2H, 2 x H(1') of dA), 6.07 and 5.90 (2 multiplets, 2H, H(1') of dihydrothymine and pyrimidinone deoxynucleoside moieties), 6.0-5.0 (broad signals, exch. D_2O, OH groups), 4.62 (s, 1H, dihydrothymine H(6)), 4.6-1.8 (complex multiplets, deoxyribose ring protons), 2.09 (s, 3H, pyrimidinone CH_3), 1.44 (s, 3H, dihydrothymine CH_3). The FAB mass spectra of the ice photoproduct are discussed in the main text.

Acid and enzymic hydrolyses of the d(T-A) ice photoproduct. The identities of the individual hydrolysis products isolated in these studies were established by comparing their UV spectra at different pH values and their mobility on TLC (in at least three different solvent systems) with those of authentic material.

Hydrolysis of the ice photoproduct with 1 M HCl, at 100°C for 4 h, gave two UV-absorbing products that were separated by ascending paper chromatography in 2-butanol saturated with water. The faster moving (R_f0.74) species was identified as adenine and the fluorescent, slower moving (R_f0.30) species as 6-(5'-methyl-2'-oxopyrimidin-4'-yl)thymine (14). Exhaustive digestion of the photoproduct with snake venom phosphodiesterase gave 5'-dAMP and a fluorescent product which remained at the origin on electrophoresis at pH 9.0. Its fluorescence characteristics (λ_{max} (excitation) at pH 7: 320 nm, λ_{max} (emission): 389 nm) closely resembled those of the parent photoproduct and it was identified as the thymidine (6-4) bimolecular photoadduct (15). Exhaustive digestion of the ice photoproduct with spleen phosphodiesterase gave 2'-deoxyadenosine and a fluorescent species which migrated more rapidly (mobility 5.8 cm $kV^{-1}h^{-1}$) than the parent photoproduct (mobility 4.1 cm $kV^{-1}h^{-1}$) on electrophoresis at pH 9.0. The UV spectrum of this digestion product, at pH 7, had maxima at identical wavelengths (259 and 319 nm) to that of the ice photoproduct; however, the A_{259}/A_{319} ratio for the digestion product (2.7) was approximately half that of the ice photoproduct (4.6). Acid hydrolysis (as above) of the fluorescent digestion product yielded adenine and 6-(5'-methyl-2'-oxopyrimidin-4'-yl)thymine (14). Its structure is discussed in the main text.

Syntheses

Imidazo[4,5-*b*]pyridine *N*(4)-oxide (3). A solution containing imidazo-[4,5-*b*]pyridine (1) (250 mg) in 90% formic acid (7.5 ml) and 30% w/v

hydrogen peroxide (5 ml) was left at room temperature for 64 h. Then a further 1 ml of hydrogen peroxide was added and the solution was maintained at 60°C for 4 h. The residue obtained on evaporation to dryness was redissolved in hot water and decolorized with charcoal. The crude product (150 mg, 53%), which separated on standing at 4°C, was recrystallised from water to give colourless needles, m.p. 270°C dec. (lit.[12], 250-253° dec.). Found: C, 53.0; H, 3.8; N, 30.8. Calc. for $C_6H_5N_3O$: C, 53.3; H, 3.7; N 31.1%. TLC: R_f (B) 0.23, (D) 0.50. UV: λ_{max} (pH 7) 216 nm (ε 26300), 294 (12000) and 303 sh (10200). ^1H NMR (D_2O, 90 MHz) δ 8.67 (s, 1H, H(2)), 8.53 (d, J 6.4 Hz, 1H, H(5)), 8.19 (d, J 8.3 Hz, 1H, H(7)), 7.64 (dd, J 6.6 and 8.3 Hz, 1H, H(6)). Mass spectrum: *m/z* 135 (M^+, 19%), 119 (M-O, 100%).

Imidazo[4,5-*b*]pyridin-5-one (5). 150 ml batches of an aqueous solution (1 mg/ml) of imidazo[4,5-*b*]pyridine *N*(4)-oxide were irradiated in a quartz tube at 254 nm (PCQ-X1 Photochemical Lamp) under nitrogen. Irradiation was continued until no further increase in the absorbance at 320 nm occurred. 1200 ml of irradiated solution was concentrated to 40 ml and on standing at 4°C deposited the crude product (381 mg, 32%) as a dark brown solid. This was repeatedly decolorized with charcoal and crystallised from water to give off-white microcrystals which analysed as the monohydrate. Found: C, 47.5; H, 4.5; N, 27.7. Calc. for $C_6H_5N_3O.H_2O$: C, 47.1; H, 4.6; N, 27.4%. On heating, compound (5) darkened and decomposed above 300°C (lit.[13,14], 311-313° dec., 318-324°). It is slightly photosensitive: aqueous solutions develop a yellow coloration on exposure to sunlight. TLC: R_f (B) 0.43, (D) 0.64, (E) 0.29. UV: λ_{max} (pH 1) 291 nm (ε 10600), 313 sh, 382 sh; (pH 7) 322 (14350); (pH 13) 318 (11800). Corrected fluorescence (pH 7): λ_{max} (excitation) 324, λ_{max} (emission) 370 nm. ^1H NMR (Me_2SO-d_6) δ 7.99 (s, 1H, exch. D_2O (on heating at 100°C for 4h), H(2)), 7.74 (d, J 9.0 Hz, 1H, H(7)), 6.27 (d, J 9.0 Hz, 1H, H(6)). Mass spectrum: *m/z* 135.0435 (M^+, 100%), calc. for $C_6H_5N_3O$ *m/z* 135.0433; *m/z* 108.0327 (M-HCN, 32%), calc. for $C_5H_4N_2O$ *m/z* 108.0330; *m/z* 107.0482 (M-CO, 35%), calc. for $C_5H_5N_3$ *m/z* 107.0483.

2-Amino-3-nitro-5-methylpyridine. This was prepared by nitration of 2-amino-5-methylpyridine using the procedure described by Vaughan et al[15]. Recrystallisation from aqueous ethanol gave yellow needles m.p. 191°C (lit.[16,17], 190-191°C). UV: λ_{max} (EtOH) 397 nm (ε 6700). ^1H NMR (Me_2SO-d_6, 90 MHz) δ 8.23 (m, 2H, H(4) and H(6)), 7.62 (bs, 2H, NH_2), 2.22 (d, J 0.5 Hz, 3H, CH_3). Mass spectrum: *m/z* 153 (M^+, 100%), 107 (M-NO_2, 31%).

2,3-Diamino-5-methylpyridine. 2-Amino-3-nitro-5-methylpyridine was reduced according to Petrow and Saper[18] as recommended by Brooks and Day[17]. Vacuum sublimation of the dried reaction mixture gave 2,3-diamino-5-methylpyridine as a tacky white solid. This was resublimed to afford chromatographically and spectroscopically (^{1}H NMR) pure material that was used in the next stage of the synthesis. UV: λ_{max} (EtOH) 246 and 315 nm. ^{1}H NMR (D_2O) δ 7.39 (m, 1H, H(6)), 6.99 (m, 1H, H(4)), 2.13 (d, J 0.6 Hz, 3H, CH_3). Mass spectrum: *m/z* 123 (M^+, 100%).

6-Methylimidazo[4,5-*b*]pyridine (2). 2,3-Diamino-5-methylpyridine was cyclized with triethyl orthoformate as described by Brooks and Day[17]. The product was crystallised from xylene, m.p. 147-148°C (lit.[17], 146-147°C). TLC: R_f (B) 0.38, (E) 0.56. UV: λ_{max} (pH 7) 244 nm (ε 4400), 287 (14300). ^{1}H NMR (D_2O) δ 8.17 (s, 1H, H(2)), 7.99 (d, J 1.6 Hz, 1H, H(5)), 7.54 (dd, J 0.7 and 1.7 Hz, 1H, H(7)), 2.28 (s, 3H, CH_3). Mass spectrum: *m/z* 133 (M^+, 100%).

6-Methylimidazo[4,5-*b*]pyridine *N*(4)-oxide (4). This was synthesised in 45% yield from 6-methylimidazo[4,5-*b*]pyridine (2) using essentially the same procedure as described above for the preparation of the unsubstituted *N*-oxide (3). Crystallisation from water gave (4) as colourless microcrystals m.p. 284-285°C. Found: C, 56.3; H, 4.8; N, 28.0. Calc. for $C_7H_7N_3O$: C, 56.4; H, 4.7; N, 28.2%. TLC: R_f (B) 0.20, (E) 0.09. UV: λ_{max} 217 nm (ε 29700), 279 (9400), 300 (10800), 310 (9900). ^{1}H NMR (D_2O) δ 8.32 (s, 1H, H(2)), 8.13 (s, 1H, H(5)), 7.71 (s, 1H, H(7)), 2.40 (s, 3H, CH_3). Mass spectrum: *m/z* 149 (M^+, 20%), 133 (M-O, 100%).

6-Methylimidazo[4,5-*b*]pyridin-5-one (6). A stirred aqueous solution (250 ml) of 6-methylimidazo[4,5-*b*]pyridine *N*(4)-oxide (4) (250 mg) was irradiated, at 254 nm under nitrogen, with the PCQ-X1 Photochemical Lamp until no further increase in absorbance at 322 nm occurred. The irradiated solution was concentrated to 10 ml and on standing at 4°C deposited 85 mg (34%) of crude product. This was decolorized with charcoal and crystallised from water to give compound (6) as colourless microcrystals which darkened and decomposed on heating above 280°C. Found: C, 56.1; H, 4.8; N, 28.0. Calc. for $C_7H_7N_3O$: C, 56.4; H, 4.7; N, 28.2%. TLC: R_f (A) 0.59, (B) 0.43, (D) 0.72, (E) 0.44. UV: λ_{max} (pH 1) 299 nm (ε 11600), 314 sh, 328 sh; (pH 7) 322 (14800); (pH 13) 321 (12600). Spectroscopic pKs 2.9 and 10.3. Corrected fluorescence (pH 7): λ_{max} (excitation) 326, λ_{max} (emission) 376 nm. ^{1}H NMR (Me_2SO-d_6) δ 12.7-11.5 (vb, 2H, exch. D_2O, H(3) and H(4)), 7.86 (s, 1H, exch. D_2O (on heating at 100°C for 4 h), H(2)), 7.61 (d, J ∿ 1Hz, 1H,

H(7)), 2.05 (d, J ∿ 1Hz, 3H, CH_3). ^{13}C NMR (Me_2SO-d_6 with tetramethylsilane as reference, 100 MHz) δ 161.6 C(5), 142.5 C(3a), 137.8 C(2), 125.3 C(7a), 120.6 C(7), 116.0 C(6), 16.7 CH_3. Mass spectrum: *m/z* 149 (M^+, 100%).

6-(5'-Methyl-2'-oxopyrimidin-4'-yl)thymine (14). Sub-milligram amounts of this compound were prepared by slight modification of the method described by Varghese and Wang[19]. Thymine was irradiated at 254 nm in frozen aqueous solution. After thawing, the solution was re-irradiated at 254 nm to convert thymine photodimer back into thymine. The fluorescent thymine photoadduct 5-hydroxy-6-(5'-methyl-2'-oxopyrimidin-4'-yl)dihydrothymine was then isolated by preparative paper chromatography in solvent B and subsequently dehydrated to compound (14) by treatment with 0.5 M HCl at 100°C for 90 min. The product was isolated by paper chromatography in solvent B; its UV spectra at pH 1 (λ_{max} 256 and 322 nm) and pH 13 (λ_{max} 301 nm) corresponded very closely with the published spectra[19].

Thymidine (6-4) bimolecular adduct (15). Thymidine was irradiated at 254 nm in frozen aqueous solution and, after thawing, the solution was re-irradiated at 254 nm to monomerize cyclobutyl photodimers. The fluorescent bimolecular photoadduct (Td_1) was then isolated by paper chromatography according to Varghese[20]. The UV spectra of the purified photoadduct at pH 1 (λ_{max} 317 nm) and pH 13 (λ_{max} 312 nm) were in excellent agreement with the published spectra[20].

RESULTS AND DISCUSSION

Preparation and characterisation of the photoproduct TA*

When d(T-A) is irradiated at 254 nm in neutral aqueous solution there are marked changes in the UV and CD spectra (Fig. 1) accompanying its conversion into the photoproduct TA*. The presence of an isodichroic point at 260 nm in successive time-course CD spectra (Fig. 1) and of isosbestic points at 222, 236 and 290 nm in the corresponding UV spectra indicates that a specific photochemical reaction is taking place. The photoproduct can be separated from unchanged d(T-A) by preparative paper chromatography and then purified to electrophoretic and chromatographic homogeneity by elution with water from a column of Sephadex G-10.

The molecular mass and elemental composition of TA* were determined by high resolution mass spectrometry of the trimethylsilylated photoproduct[21]. The most prominent ion in the high mass range had *m/z* 915.3438 corresponding to $C_{35}H_{66}N_7O_{10}PSi_5$ (calc. *m/z* 915.3453); a shift of 45 mass units to *m/z* 960 in the spectrum of the perdeuterotrimethylsilyl derivative

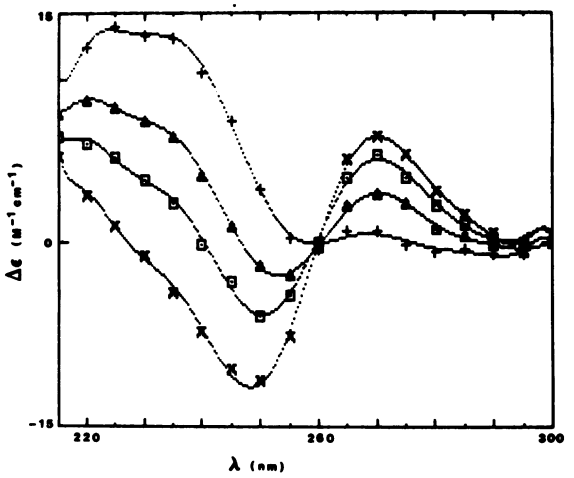


Fig. 1. Variation of the CD spectrum of d(T-A) as a function of UV fluence. A 3 x 10^{-5} M solution of d(T-A) in pH 7.0 buffer was irradiated at 254 nm and its CD spectrum was recorded after 0 (X), 15 (⊡), 30 (△), and 60 min (+). For each spectrum the CD signal was measured at intervals of 5 nm over the range 215-300 nm. These data points were used to generate the spectra shown in the Figure. The spectra were computed using the curve fitting procedure, based on Chebychev polynomials, described in reference 23. The programme was run on an Apple II microprocessor.

confirmed the presence of 5 TMS groups. The deduced composition of TA*, $C_{20}H_{26}N_7O_{10}P$, shows that it is isomeric with d(T-A) and must therefore result from an intramolecular photorearrangement.

It is clear that the photoreaction involves major disruption to the conjugated π-electron systems of both the adenine and thymine bases because the UV absorption of TA* is very much weaker than that of d(T-A)[9]. This could be accounted for if TA* were formed by photoaddition of the 5,6-double bond of thymine across a π bond in the adenine nucleus to give a structure such as that shown in Fig. 2. Support for this interpretation

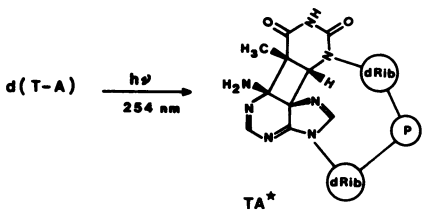


Fig. 2. Possible structure for the d(T-A) photoproduct, TA*.

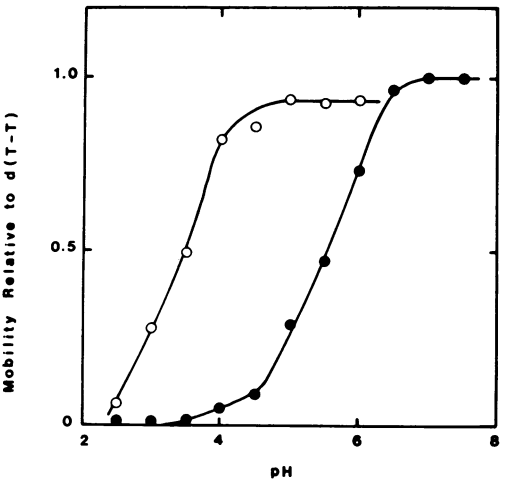


Fig. 3. Electrophoretic determination of the pK for protonation of d(T-A) and TA*. The electrophoretic mobility of each compound was measured relative to that of d(T-T) in appropriate buffers covering the pH range 2.5 to 7.5: (○), d(T-A); (●), TA*. At all pH values investigated d(T-T) bears a single negative charge.

comes from the ^{1}H NMR spectrum of TA* where the signals for the base protons and thymine methyl group are shifted to higher field than the corresponding signals[22] in d(T-A). The absence of any splitting due to allylic coupling in the methyl resonance of TA*, and the occurrence of a base proton singlet at δ 5.03, are both consistent with saturation of the thymine 5,6-double bond. Moreover, the signal patterns for the deoxyribose ring protons in d(T-A) and TA* show only small differences suggesting that the deoxyribose-phosphate backbone is unaffected by the photoreaction. Partial saturation of the adenine ring in TA* is also indicated by the increased basicity of its amino function. The pK for protonation was estimated as 5.5 for TA*, compared with 3.5 for d(T-A), from the pH dependence of its electrophoretic mobility measured relative to d(T-T) as standard (Fig. 3).

The feasibility of photocycloaddition reactions between thymine and a purine base has been demonstrated in dinucleotide analogues in which a trimethylene bridge links N(1) of thymine to N(9) of either adenine[24] or hypoxanthine[25]. UV irradiation of the adenine-containing analogue leads to an azacyclobutane photoproduct formed by cycloaddition between the 5,6-double bond of thymine and the N(7)-C(8) double bond of adenine. This photoproduct is, however, distinctly different from TA* because (i) it has an absorbance maximum at 284 nm, (ii) it reverts to starting material on

irradiation at 254 and 290 nm whereas TA* is unaffected by these wavelengths. With the hypoxanthine-containing dinucleotide analogue the thymine 5,6-double bond undergoes photoaddition across the central 5,4-double bond of the purine ring. Again, the resultant photoproduct differs from TA* in that it is cleaved by 254 nm radiation. Presumably, therefore, TA* is generated via some other mode of photoaddition between thymine and the adenine nucleus. One possibility which avoids the formation of carbon-nitrogen bonds and appears to be stereochemically reasonable if d(T-A) adopts a stacked conformation in solution[22], involves coupling of the thymine C(5) and C(6) atoms to C(6) and C(5) of adenine respectively, as shown in Fig. 2. As discussed below, the behaviour of TA* on acid hydrolysis can also be rationalised in terms of this structure. On this basis, and in view of its general compatibility with the properties of TA*, it can be advanced as the most plausible candidate structure for the photoproduct.

Acid Hydrolysis of TA*

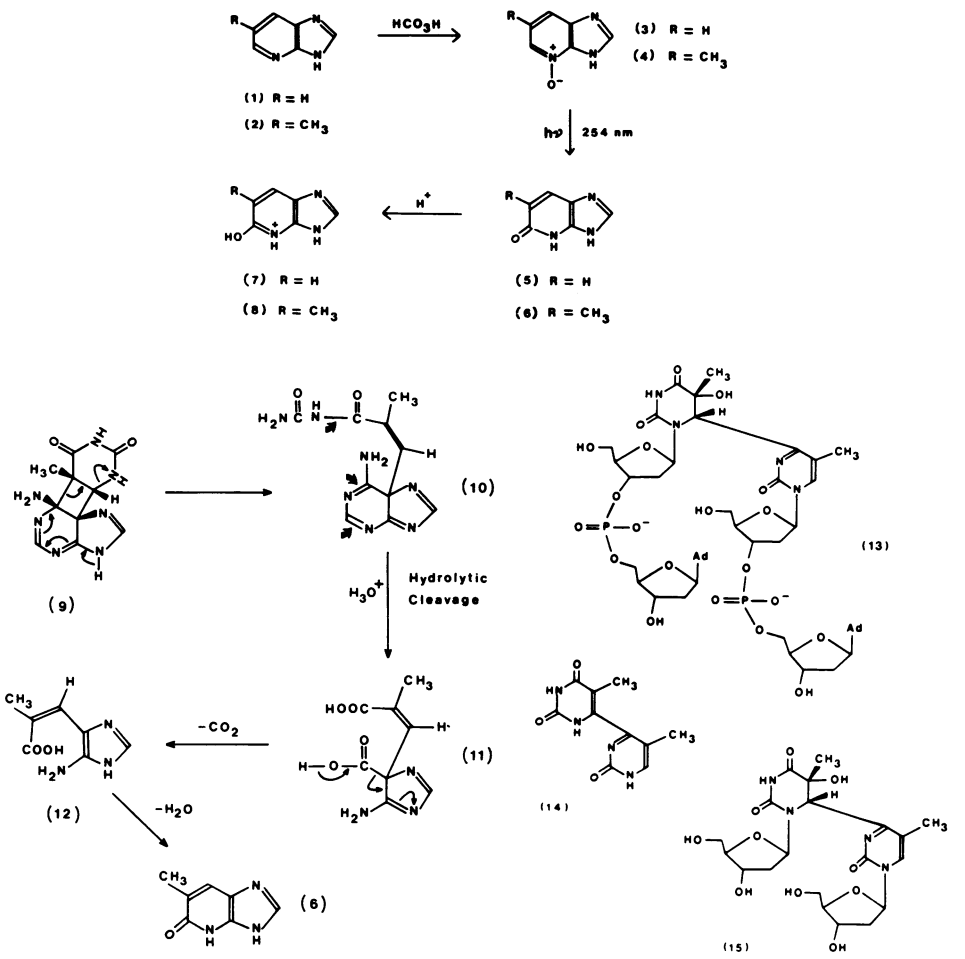
Attempts to elucidate the structure of TA* by enzymatic degradation to the nucleoside level were unsuccessful because the photoproduct is resistant to cleavage by the phosphodiesterase enzymes from snake venom and bovine spleen. However, acid hydrolysis (1M HCl, 100°C, 4 h) led to the isolation of a fluorescent modified base whose molecular formula was determined as $C_7H_7N_3O$ by high resolution mass spectrometry. The spectroscopic properties of the modified base could not be matched with those previously recorded for any simple purine or pyrimidine derivatives. In particular, the compound showed minimal UV absorption in the region of 250-260 nm while the absorbance maximum shifted from 322 nm at pH 7 to 299 nm at pH 1. The ^1H NMR spectrum showed signals for two aromatic protons. One of them exchanged with D_2O at 100°C indicating the probable presence of an imidazole ring[26]. The other was allylically coupled to the signal for a methyl group. From a detailed assessment of the structural possibilities, the modified base was tentatively identified as a *C*-methyl derivative of the imidazol[4,5-*b*]pyridin-5-one (1-deazapurin-2-one) ring system.

Although the parent heterocycle (5) has been synthesised previously[13, 14] (and patented as an antifoggant in diffusion transfer photographic processes[14]) no information is available concerning its spectroscopic properties. To prepare compound (5) we devised a new synthetic route from (commercially available) imidazo[4,5-*b*]pyridine (1). Treatment of (1) with

performic acid gives the known[12] *N*(4)-oxide (3) and this on irradiation at 254 nm in aqueous solution rearranges to imidazo[4,5-*b*]pyridin-5-one (5). Similar photorearrangements of other heterocyclic *N*-oxides are well documented[27] including that of purine N(3)-oxide to 2-oxopurine[28].

The fluorescence and absorption characteristics of (5) corresponded very closely with those of the acid hydrolysis product derived from TA* confirming that the latter contains the imidazo[4,5-*b*]pyridin-5-one ring system. The characteristic blue shift in UV absorption at low pH parallels that observed with 2-pyridone and is presumably caused by a similar change in tautomeric form accompanying protonation as illustrated in structures (7) and (8). By comparing the chemical shifts of the aromatic protons in the ^{1}H NMR spectra of (5) and the acid hydrolysis product of TA* the methyl substituent in the latter compound could be assigned to the C(6) position. The protons at C(6) and C(7) in the pyridone ring of (5) form an AB system and have chemical shifts of δ 6.27 and 7.74 respectively; as observed for simple 2-pyridones[30], and other ring systems containing an α,β-unsaturated carbonyl function, the proton on the carbon atom adjacent to the carbonyl group resonates at markedly higher field. As the signal for the pyridone ring proton in the acid hydrolysis product occurs at δ 7.61 it is clear that the methyl substituent must have replaced the hydrogen on C(6).

The identity of the acid hydrolysis product as 6-methylimidazo[4,5-*b*]-pyridin-5-one was confirmed by chemical synthesis. 6-Methylimidazo[4,5-*b*]-pyridine (2) was prepared from commercially available 2-amino-5-methyl-pyridine essentially as described by Brooks and Day[17]. The starting material was nitrated to give 2-amino-3-nitro-5-methylpyridine which was reduced to 2,3-diamino-5-methylpyridine and then cyclised by heating with with triethyl orthoformate to give (2). Spectroscopic data for these intermediates are given in the Experimental Section as they have not been reported previously. The *N*(4)-oxide (4) was prepared from (2) by oxidation with performic acid; its mass spectrum, like that of (3), exhibited a characteristically intense peak corresponding to loss of oxygen from the molecular ion[27]. On irradiation at 254 nm in aqueous solution (4) was converted efficiently into (6), the spectroscopic yield being $\sim$ 50%. By using a calibrated UV source, the quantum yield for the photorearrangement was estimated as 0.14 mol einstein^{-1} from the increase in absorbance at 322 nm as a function of UV fluence. The 6-methylimidazo[4,5-*b*]pyridin-5-one (6) isolated from the irradiated solution was identical in all respects to the fluorescent acid hydrolysis product prepared from TA*.



Scheme 1. Proposed mechanism for formation of 6-methylimidazo[4,5-*b*]pyridin-5-one (6) on acid degradation of the d(T-A) photoproduct, TA*. Solid arrows (➡), indicate assumed sites of hydrolytic cleavage in structure (10).

Mechanistic Considerations

During its conversion into (6) the photoproduct TA* evidently undergoes extensive degradation and rearrangement. This makes it very difficult to draw definitive conclusions about the structure of TA* from that of (6). When the reaction of TA* with 1M HCl at 95°C is followed by spectrophotometry, complex spectral changes, suggestive of a multi-step process, are observed which require approximately 4 h to reach completion. Nevertheless, the conversion of TA* into (6) occurs with considerable

specificity as evidenced by a spectroscopic yield of at least 53%. Some insight regarding the mechanism of the reaction is provided by experiments (described elsewhere[9,11]) with radiolabelled preparations of poly(dA-dT). These have established that when (6) is produced from the UV-irradiated copolymer it incorporates the C(8) carbon of adenine and the methyl group of thymine but the C(2) atom of thymine is not retained. From these findings it can be inferred that the imidazole ring moiety of (6) is most probably derived from adenine while the pyridone moiety including the *C*-methyl group is at least partially derived from thymine. A mechanistic scheme to account for the formation of (6) from TA* is advanced in Scheme 1. Although it is generally consistent with the experimental observations, it must be regarded as tentative until more rigorous evidence can be adduced in its favour.

In formulating the mechanism it is assumed, as discussed earlier, that TA* has the structure shown in Fig. 2. Initially, treatment of TA* with acid should remove the deoxyribose-phosphate backbone, by cleaving both glycosidic bonds, to give the parent adenine-thymine photoadduct (9). Sequential degradation of (9) is then postulated to occur as outlined in Scheme 1. First, the strained cyclobutyl ring is opened in a rearrangement involving concomitant fission of the thymine ring to generate structure (10). Acid hydrolysis of the ureido and imino functions of (10), at the positions indicated, then leads to (11) which decarboxylates to the 4,5-disubstituted imidazole (12). Finally, the exocyclic amino and 2-carboxypropenyl substituents cyclise to give (6).

The production of (6) on acid hydrolysis provides a sensitive diagnostic test for the presence of TA* in UV-irradiated oligo- and polydeoxyribonucleotides. Its distinctive UV absorption and fluorescence characteristics permit very small amounts of (6) to be detected and estimated without serious interference from other DNA photoproducts.

The quantum yield for formation of TA* from d(T-A) was determined as $\sim 7 \times 10^{-4}$ mol einstein^{-1} from the decrease in UV absorption as a function of fluence at 254 nm. The photoreaction presumably involves a singlet excited state species because it is unaffected by triplet state quenchers such as oxygen and 0.1 M Mn^{2+}. Furthermore, no reaction is observed when d(T-A) is irradiated at wavelengths >290 nm, either alone or in the presence of acetone as triplet photosensitiser.

The photoreaction is sequence-specific since UV irradiation of d(A-T) does not give a photoproduct comparable to TA*. In principle, a similar

photoreaction might occur with d(C-A) and/or d(T-G). This possibility was investigated by electrophoretic and chromatographic analysis of irradiated samples and their acid hydrolysates. In the case of d(C-A), acid hydrolysis of the analogous photoproduct might be expected to give compound (5) which would have been readily identified by comparison with synthetic material. However, no evidence for the formation of a purine-pyrimidine photoadduct was obtained with either d(C-A) or d(T-C). The lack of reaction with d(C-A) might result from preferential photohydration[4] of the cytosine base.

The d(T-A) Ice Photoproduct

The photochemical behaviour of nucleic acid components in frozen aqueous solution is often markedly different from their behaviour in normal liquid solution. This reflects a change in their physical state from isolated hydrated molecules in solution to ordered molecular aggregates dispersed in an ice matrix[31]. When d(T-A) was irradiated at 254 nm in frozen aqueous solution, the intramolecular photoadduct TA* was not produced in significant amounts. Instead, a new photoproduct was formed which was isolated by high voltage paper electrophoresis and then purified to homogeneity by elution from Sephadex G-10 with water. FAB mass spectrometry of the photoproduct showed it to be a dimer of d(T-A) having molecular weight 1110; the most abundant ions in the high mass range corresponded to MH^+, with *m/z* 1111, in the positive ion spectrum, and $(M-H)^-$, with *m/z* 1109, in the negative ion spectrum.

Initial assignment of the structure of the dimeric ice photoproduct was based on its UV absorption peak at 319 nm and the associated fluorescence emission maximum at 388 nm which compare closely with data reported for the bimolecular photoadducts of thymine and thymidine[5,20]. Accordingly, it seemed probable that the photoproduct was formed by the coupling of two d(T-A) molecules through their respective thymine bases as shown in structure (13). This was confirmed by enzymatic and acid hydrolysis of the ice photoproduct. Digestion with snake venom phosphodiesterase gave 5'-dAMP and a fluorescent species which was shown by spectroscopic and chromatographic comparison to be identical with the thymidine photoadduct (15) prepared as described by Varghese[20]. Hydrolysis with HCl gave adenine and 6-(5'-methyl-2'-oxopyrimidin-4'-yl)thymine (14). The latter results from acid catalysed dehydration of the original thymine bimolecular photo-adduct; it was identified by comparison with a sample prepared from thymine by the procedure of Varghese and Wang[19]. Exhaustive digestion with bovine

spleen phosphodiesterase cleaved only one of the two phosphodiester bonds in the photoproduct to yield 2'-deoxyadenosine and a fluorescent product having high electrophoretic mobility at pH 9.0. The latter on acid hydrolysis gave (14) and adenine. Its UV spectrum was consistent with the presence of one adenine base per molecule as its relative extinction at 259 nm was approximately midway between that of the original ice photoproduct and that of the thymidine photoadduct (15). No attempt was made to ascertain which of the two phosphodiester linkages in the ice photoproduct is resistant to hydrolysis by the bovine spleen enzyme.

The ^{1}H NMR spectrum of the ice photoproduct is fully in agreement with the proposed structure (13). Although the indicated stereochemistry about the 5,6-bond of the dihydrothymine ring cannot be deduced from our spectroscopic measurements, it is assumed to be the same as that determined for the thymine bimolecular photoadduct by X-ray crystallography[32].

The formation of a bimolecular photoadduct in frozen aqueous solution suggests that under these conditions the d(T-A) molecules are assembled into ordered aggregates whose geometry favours the observed photoreaction. This would require that the thymine bases of two adjacent d(T-A) molecules are positioned so that photoaddition can occur between the 5,6-double bond of one thymine and the C(4) carbonyl function of the other to generate an oxetane intermediate[5] which subsequently rearranges to the isolated photoadduct. It is known that evaporation of aqueous solutions of the triethylammonium salt of d(T-A) results in an ordered structure with fibre-like X-ray diffraction properties. From analysis of the diffraction pattern Radwan and Wilson[33] have proposed that the individual d(T-A) molecules are arranged in an infinite left-handed helical array stabilised by Hoogsteen base-pairing. In this structure, photoreaction between adjacent thymine bases to give the ice photoproduct does appear to be stereochemically feasible and it may, therefore, represent the way in which d(T-A) molecules are organised in an ice matrix. The same organisation evidently persists when d(T-A) solutions as dilute as 0.5 μM are frozen because the ice photoproduct is still formed at this concentration.

CONCLUSION

The deoxydinucleoside monophosphate d(T-A) exhibits quite different photochemical behaviour in neutral aqueous solution and in ice. Irradiation at 254 nm in aqueous solution gives a novel intramolecular adenine-thymine photoadduct which is degraded by acid to the fluorescent

heterocyclic base 6-methylimidazo[4,5-*b*]pyridin-5-one (6). In contrast, irradiation of d(T-A) in frozen aqueous solution leads to the dimeric photoproduct (13) which results from (6-4) photoadduct formation between the thymine bases of two d(T-A) molecules. Despite its intramolecular nature the photoreaction observed in solution does not occur to an appreciable extent in ice. Possibly, this is because the conformation of the aggregated d(T-A) molecules in an ice matrix is sufficiently different from that of d(T-A) molecules in solution to inhibit the photoreaction from taking place. Alternatively, the dimeric ice photoproduct (13) may be formed so efficiently in frozen solution that the competing intramolecular photoreaction is effectively suppressed. Both types of photoreaction occur when native DNA is irradiated at 254 nm in aqueous solution[11,34]. Accordingly, it is most probable that they both contribute to the biological effects associated with UV radiation.

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