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Thymine Photodimer Formation in DNA Hairpins. Unusual Conformations Favor (6-4) vs. (2+2) Adducts^{†,‡}

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Abstract

The photochemical reactions of eleven synthetic DNA hairpins possessing a single TT step either in a base-paired stem or in a hexanucleotide linker have been investigated. The major reaction products have been identified as the *cis-syn* (2+2) adduct and the (6-4) adduct on the basis of their spectroscopic properties including 1D and 2D NMR spectra, UV spectra and stability or instability to photochemical cleavage. Product quantum yields and ratios determined by HPLC analysis allow the behaviour of the eleven hairpins to be placed into three groups: Group I in which the (2+2) adduct is the major product, as is usually the case for DNA, Group II in which comparable amounts of (2+2) and (6-4) adducts are formed, and Group III in which the major product is the (6-4) adduct. The latter behaviour is without precedent in natural or synthetic DNA and appears to be related to the highly fluxional structures of the hairpin reactants. Molecular dynamics simulation of ground state conformations provides quantum yields and product ratios calculated using a single parameter model that are in reasonable agreement with most of the experimental results. Factors which may influence the observed product ratios are discussed.

Introduction

Irradiation of single strand or duplex DNA containing a TT step results in the formation of two major TT photoadducts, the *cis-syn* (2+2) cyclobutane adduct and the (6-4) pyrimidine-pyrimidone adduct (Scheme 1).¹⁻⁴ The (2+2) adduct is the major product obtained both from irradiation of cellular and DNA and model systems.⁵ The (6-4) adduct is formed upon thermal ring opening of an unstable oxetane intermediate which is the product of a cycloaddition reaction between the C=O double bond of the 3'-thymine to the C=C double

[†]Electronic supplementary information (ESI) available: Experimental Section. MALDI-TOF, T_M, and selected NMR data. HPLC and UV data for irradiated solutions. Calculated UV spectra and quantum yield data. See DOI: 10.1039/b000000x/

[‡]Dedicated to the memory of Nicholas J. Turro, Educator and Scientist Extraordinaire

bond of its 5'-neighbor.² Formation of either adduct requires DNA unwinding and base-rotation in order to bring the reactive double bonds into conformations favourable for bond formation. Thus TT adduct formation occurs more readily in premelted and curved DNA tracts and less readily in triple stranded DNA.⁶ Formation of (6-4) adducts is disfavoured relative to (2+2) formation adduct within nucleosome cores, adjacent to purine bases in short duplex sequences,⁷ and in peptide nucleic acids⁸ and locked nucleic acids.^{9, 10}

Whereas base sequences and modifications which disfavour (6-4) adduct formation are known; to our knowledge there are no natural or unnatural base sequences in which (6-4) adduct formation is the major photoproduct. Thus we were surprised to observe similar yields of (2+2) and (6-4) TT adduct formation (55% and 45%, respectively) from the synthetic hairpin **H₆3** (Chart 1) in which the TT step is located adjacent to dodecane a (C12) linker.¹¹ Subsequent NMR structure determination for the isomeric hairpin **H₆2** revealed a slightly distorted base pair adjacent to the linker, providing a possible explanation for the product ratio based on a non-standard structure.¹²

A search for further examples of high yields of (6-4) TT adduct formation led us to examine two additional classes of synthetic hairpins, the C12-linked hairpins in the **H₄** series and the hexanucleotide (hexaloop) hairpins in the **L₆** series (Chart 1). The **H₄** hairpins have low melting temperatures and thus were expected to mimic the behaviour of duplexes under premelting conditions. The choice of the **L₆** series was based in part on a report by Carell et al. of high yields of a complex mixture of products upon irradiation of a hairpin having a T₄ tetraloop.¹³ Hexaloops were selected to provide a greater variety of TT dimer sites than provided by tetraloops. The results for these two classes of hairpins are reported herein along with results for hairpins possessing six A-T base pairs (**H₆1-H₆3**) and the single strand oligonucleotide **SS-1** (Chart 1).¹⁰ The experimental results are compared with calculated quantum yields and product ratios obtained from conformational modelling of the (2+2) and (6-4) reactions using single-parameter models developed during our earlier studies of TT photodimerization.¹⁴

Results and Discussion

Hairpin Structures

Methods for the synthesis, purification, and characterization of dodecane (C12)-linked hairpins have been previously reported.¹⁵ MALDI-TOF mass spectra for the C12-linked hairpins are reported in Table S1[†] along with values for the melting temperatures (T_M) determined from the derivatives of thermal dissociation profiles (Fig. S1[†]) for both the C12-linked and hexaloop hairpins. Hairpins **H₆1-H₆3** and **H₄1-H₄3** have values of T_M between 49-51 °C and 25-27 °C, respectively, in potassium phosphate pH 7.2 buffer containing 100 mM NaCl. The T_M values for the hexaloop hairpins **L₆1-L₆3** are lower than those for a C12-linked hairpins possessing the same base pair stem.

[†]Electronic supplementary information (ESI) available: Experimental Section, MALDI-TOF, T_M , and selected NMR data. HPLC and UV data for irradiated solutions. Calculated UV spectra and quantum yield data. See DOI: 10.1039/b000000x/

The solution structure of hairpin **H₆2** obtained from ¹H NMR data with restrained molecular dynamics has previously been reported.¹² To briefly summarize, the C12 linker adopts a highly curved structure which appears to maximize hydrophobic interactions with the adjacent base pair at the expense of a shortened P-P distance and some distortion of normal Watson-Crick base pair geometry. The terminal base pair is extensively end-frayed, whereas the four internal base pairs display normal B-DNA geometry, thus providing a good model for longer A-T base pair domains. The average structure of the base pair adjacent to the linker has a larger buckle than those for the other five base pairs (22° vs. <|6°|).

The solution structure of hairpin **H₄2** has been investigated using the methods employed for **H₆2**.¹² The 1D NMR spectra of **H₄2** in the imino proton region (δ13-14.5 ppm) recorded at temperatures between 5-15°C are shown in Fig. S2[†]. Two of the four imino protons can be observed at 5 °C, but not at higher temperatures. Evidently, rapid exchange of the imino protons compared to the NMR time scale occurs well below the hairpin UV melting temperature, as is the case for **H₆2** and, more generally for oligonucleotide duplex DNA.¹² The 2D NOESY spectra for **H₄2** is shown in Fig. S3[†] and the chemical shifts for the base protons are reported in Table S2[†]. The chemical shifts for some of these protons show substantial changes upon heating (Fig. S4[†]). Derivatives of the temperature dependence of the chemical shifts provide an average NMR melting temperature similar to the value determined by UV melting (26 °C).

The 1D spectra of **H₄2** in the aliphatic proton region 2-0 ppm recorded at temperatures between 5-80 °C are shown in Fig. 1. The aliphatic protons from C2-C11 of the dodecyl-linker and the thymine methyl signals can be seen in this spectral region. The protons next to the phosphate at C1 and C12 have chemical shifts between 3.4-3.9 ppm (region not shown). Eight clearly distinguishable signals for the protons of the C12 linker are observed at temperatures above T_M (26 °C), indicative of the proximity of the linker to the DNA bases. At low temperatures the signals of **H₄2** are also broadened, an effect attributed to dynamic movement of the linker and bases on the NMR timescale during base-pair melting and the averaging of the signals from the different conformations. The region of linker-linker cross-peaks in the 2D NOESY, TOCSY and COSY spectra of **H₄2** is crowded (compare Fig. 1), but the signal dispersion allows assignment of the protons H1 to H5 and H10 to H12 of the linker (Table S3[†]). Partial assignments for the C12 linker protons in hairpin **H₄2** indicate that the hairpin loop adopts a structure similar to that of **H₆2**.

The upfield region of the 1D NMR spectrum of **H₆3** is shown in Fig. 2 along with the spectra of its (2+2) and (6-4) photoproducts. The thymine methyl singlets and most of the linker methylene protons have signals in this region. Assignments of the thymidine methyl singlets for **H₆3** obtained from NOESY and TOCSY spectra (Fig. S5[†]) are shown in Fig. 2a.

The structures of **L₆1-L₆3** were not investigated by solution NMR methods. A previous NMR study of the conformation of DNA hairpin d(CGATTTCG-T₄-CGATCG) showed that both the phosphate backbone between T2-T3 in the loop and C7 and G8 in the stem display exceptional flexibility, undergoing rapid exchange between conformers.¹⁶ An NMR study of the conformation of the hairpin d(ATCCTA-GTTA-TAGGAT) showed that the tetraloop has a sheared G-A base pair on top of the Watson-Crick six base-paired stem and that T8

stacks upon its 5'-neighboring G7; whereas T9 is directed away from the loop and thus does not stack with T8.¹⁷

Photochemical Reactions

We previously reported preliminary results for the irradiation of hairpins **H61-3**.¹¹ HPLC analysis of solutions of **H61** and **H62** revealed the formation of a single product peak identified as the *cis-syn* (2+2) adduct upon irradiation with monochromatic 280 nm light; whereas irradiation of **H63** yields the (6-4) as well as the (2+2) adduct, as shown in Scheme 2. The structure assignment of the (2+2) adduct from **H63** is supported by analysis of the 1D and 2D ¹H NMR spectra. Sequential assignments of the base and H1' protons, including the six thymine methyl singlets obtained from NOESY spectra are shown in Fig. S6[†]. The chemical shifts of the cyclobutane methyl protons (1.42 and 0.74 ppm, Figure 2b) are similar to those for (2+2) adducts embedded in duplexes flanked by guanines or adenines.^{18, 19} The assignment of the structure of the (6-4) adduct from **H63** is also supported by analysis of 1D and 2D ¹H NMR spectra (Fig. S7[†]). The NMR assignments shown in Figure 2c are in general agreement with those of Kim and Choi for a (6-4) adduct.²⁰

All of the photoproducts formed by the hairpins in Chart 1 have the same mass as the starting material. Where NMR data was not available, product identification was based on the presence of a weak absorption band at 320 nm for the (6-4) adduct but not the (2+2) adduct and the rapid reversion of the (2+2) adduct but not the (6-4) adduct upon irradiation at 240 nm (Scheme 1). HPLC traces of solutions of **H61-3**, **H41-3**, and **L61-3** obtained at several irradiation times along with the UV spectra of the (2+2) and (6-4) adducts are provided in Figs. S8-S16[†] and the calculated spectra of the (2+2) and (6-4) adducts of the dinucleotide T_pT are shown in Fig. S17[†].

Relative yields for product formation were determined from the initial slopes of plots of product yield vs. irradiation time.¹¹ Relative yields were converted to the quantum yields reported in Table 1 using ferrioxalate actinometry to determine light intensities. Quantum yields are not corrected for competitive absorption by non-reactive nucleobases. In all cases the total quantum yield is <0.02, indicative of inefficient adduct formation.

The behaviour of the hairpins in Chart 1 can be divided into three groups determined by the ratio of quantum yields Φ_{2+2}/Φ_{6-4} (*R*). Hairpins in group I (**H61**, **H62**, **2H6**, **L63**, and **SS1**) have values of *R* > 10, similar to the values for the duplex dT₂₀dA₂₀ and the single strand dT₂₀,¹⁴ as well as native DNA duplexes.⁵ Hairpins in group II (**H42**, **2H4**, **H63**, **L61**, and **L62**) have values of *R* = 1-2 and hairpins in group III (**H41** and **H43**) have values of *R* < 1. To our knowledge these hairpins provide the first examples of (6-4) adduct formation as the major product of TT photoaddition. We note that hairpins **H42** and **2H4** as well as **H62** and **2H6** belong to the same groups, even though they differ in the polarity of their TT steps.

Calculated Quantum Yields

The efficiency of TT dimerization in both DNA and small model oligonucleotides is low, in spite of the ultrafast nature of the dimerization reaction.²¹ The possibility that the products and efficiency of TT dimerization in DNA are related to ground-state conformation as well

as base sequence was proposed over 40 years ago.²²⁻²⁴ Law et al.²⁵ and Johnson et al.²⁶ proposed models based on the ground state conformations of (dT)₂ and (dT)₁₈, respectively, in which dimerization only occurs when the distance and dihedral angle between C=C double bonds are within prescribed limits.

We previously advanced a simple empirical single parameter model for adduct formation in both single strand and duplex DNA based only on the distance d between the centers of C=C bonds for (2+2) adduct formation and the distance g between the carbonyl oxygen of the 3'-thymine and the C5 end of the C=C bond of the 5'-thymine for (6-4) adduct formation.¹⁴ Theoretical modelling of the excited state (2+2) reactions showed that the primary constraint on dimerization is the distance and not the dihedral angle between the two double bonds.¹⁴ Values of $d < 3.52$ Å and $g < 2.87$ Å were selected based on fits to experimental data for (dT)₂, (dT)₂₀ and (dA)₂₀(dT)₂₀. We note that both the average distance and the cutoff for oxetane formation are shorter than those for (2+2) cycloaddition. Values of Φ_{2+2} calculated using molecular dynamics simulations and this value of d for several XTT and TTX trinucleotides²⁷ and for single strand and hairpin systems possessing TT steps in natural and locked nucleic acids¹⁰ are in fair to good agreement with experimental values. Experimental data for (6-4) adduct formation is more limited and calculated quantum yields are only in fair agreement with this data.¹⁴

Calculated quantum yields for (2+2) and (6-4) adduct formation from **H₄1-3** and **L₆1-L₆3** were obtained as previously described for **H₆1-3**.¹⁴ Probability densities for distances d and g in the hexaloop hairpins **L₆1-3** are shown in Fig. 3. These plots have maxima at distances < 5 Å for hairpins **L₆1** and **L₆2**; whereas the plots for **L₆3** do not. The probability densities for g show shorter distance distributions for interaction of the 3'-C=O with 5'-C=C than vice versa, as expected for (6-4) adduct formation. The zero probability density of reactive geometries for **L₆3** suggests that the central TT step in the hexaloop may exist in an orthogonal geometry similar to that reported for a GTTA tetraloop.¹⁶

Calculated quantum yields for (2+2) and (6-4) adduct formation for a range of cutoff values for d and g are shown in Fig. 4 for **L₆1-3**. Using values of $d < 3.52$ Å and $g < 2.87$ Å obtained in our earlier study¹⁴ provides the calculated quantum yields reported in Table 1 for the hexaloop hairpins as well as for the other hairpins in Chart 1. Our simple one-parameter model for (2+2) and (6-4) TT dimer formation provides quantum yields for hairpin adduct formation in reasonable agreement with the experimental results. The model correctly predicts the relative order of reactivity for the three hexaloop hairpins and provides values of R (Table 2) that are in the same group as the experimental values for six of nine hairpins (Table 1). Calculated values of R are significantly larger than experimental values the case of Φ_{2+2} for **L₆1** and **L₆2**; whereas the calculated value of Φ_{6-4} is too small for **H₄2**. Small changes in the values of d or g do not appreciably change the agreement between experimental and calculated quantum yields or values of R .

Conclusions

The hairpins **H₄1** and **H₄3** provide the first examples of DNA constructs possessing a TT step in which the (6-4) adduct rather than the *cis-syn* (2+2) adduct is the major photoproduct

(Table 1). Several other hairpins studied in the present investigation yields comparable amounts of (6-4) and (2+2) adducts. A report selective formation of a (2+2) adduct from an LNA dinucleotide was attributed to the fixed C3'-endo conformation of the LNA sugar and the absence of (6-4) adduct offered as evidence for formation from the minor C2'-endo deoxyribose conformation DNA. A recent computational study of the photoaddition reactions of the TpT dinucleotide provided further support for the formation of the (2+2) and (6-4) adducts from the C3'-endo C2'-endo sugar conformations, respectively.²⁸

The observation of low values of R for hairpins with either low R melting temperatures (**H₄** hairpins) or unusual base pairing stacking motifs (**L₆₁**, **L₆₂** and **H₆₃**) suggests that (6-4) adduct formation is favoured by flexible, stacked conformations different from those found as the major conformers in either single strand or B-form duplex DNA. Unfortunately such highly flexible hairpins are not readily amenable to full structure determination by solution NMR methods. Nor is it certain that the photoproduct yields can be related to the major ground state conformations. Calculated quantum yields and product ratios obtained using empirical one-parameter model for (2+2) and (6-4) adduct formation¹⁴ provide fair to good agreement with experimental results for some, but not all of our hairpins (Tables 1 and 2). However, our model specifies only the distance between adjacent thymine's and does not specify sugar conformation or require π -stacking of adjacent thymine's. Furthermore, it is parameterized for hairpins possessing only A-T base pairs thus is not expected to be applicable to hairpins or duplexes which the reactive TT step has flanking purine bases. We and others have observed that quenching of (2+2) adduct formation by a neighboring purine bases are related to purine oxidation potential.⁷ Furthermore, quenching of oxetane formation purines is more pronounced than quenching of cyclobutane formation. Our empirical model also does not provide information about the excited states involved in (6-4) and (2+2) adduct formation.

Markovitsi and Improta and co-workers have recently proposed that (2+2) adduct formation occurs from a singlet exciton; whereas oxetane formation proceeds from a charge transfer excited state.^{28, 29} The wavelength dependence of the product ratio for hairpins such as **H₆** might be useful in testing this proposal. Giussani et al. have recently investigated mechanism of (6-4) adduct formation for thymine dimerization using a CASPT2//CASSCF approach.³⁰ They propose formation of the (6-4) adduct occurs from the triplet $^3(n,\pi^*)$ state formed via intersystem crossing of the $^1(n,\pi^*)$ state; whereas (2+2) adduct formation occurs via the bright $^1(\pi,\pi^*)$ state. Most intriguing is their proposal that $n \rightarrow \pi^*$ excitation is favoured by conformations in which the two thymine's of the dinucleotide are poorly stacked. Further experimental and theoretical studies will be required to fully elucidate the ground state conformational factors and excited state pathways that define thymine photo-dimerization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Temperature dependent 1D NMR spectra for the upfield region of **H₄₂**. Linker and thymine methyl assignments are reported in Tables S2 and S3[†]

Fig. 2.
 (a) Upfield region of the 1D ^1H NMR spectra of $\text{H}_6\text{3}$ and its photoproducts (b) the (2+2) adduct and (c) (6+4) adduct recorded at 20 $^\circ\text{C}$ (see Chart 1 for numbering of thymidine methyl groups)

Fig. 3.
Normalized probability densities for (a) d and (b) g calculated from MD trajectories for the hairpins L_{61} - L_{63}

Fig. 4. Calculated quantum yields from molecular dynamics simulations for hairpins **L₆1** (blue) and **L₆2** (green) and **L₆3** (red). (a) (2+2) adduct formation vs. cutoff value for d_{max} and (b) (6-4) adduct formation vs. cutoff value for g_{max} (values for dashed lines too small to detect)

Scheme 1.

Formation of (2+2) and (6-4) adducts from irradiation of DNA containing a TT step

Scheme 2.

Snapshots of structures from MD trajectories with geometries favorable for formation of a thymine-thymine (2+2) (left) and (6-4) adduct (right) within the **H₆₃** hairpin. The inter-base bonds are added artificially to help guide the reader's eye.

Chart 1.

Structures of DNA hairpin conjugates possessing 4 and 6 AT base pairs and either a dodecane (C12) linker or a hexaloop (TT step is shown in blue, bases of **H₆3** are numbered from the 5' end).

Table 1

Experimental and calculated quantum yields for (2+2) and (6-4) adduct formation and ratios for (2+2)/(6-4) adduct formation (*R*)

hairpin	$10^{-3}\Phi_{2+2}$		$10^{-3}\Phi_{6-4}$		<i>R</i>	
	exp	calcd	exp	calcd	exp	calcd
H₆1	0.39	1.5	<i>a</i>	0.3	>20	5
H₆2	0.92	1.5	<i>a</i>	0.3	>20	5
H₆3	1.1	0.9	0.76	1.4	1.4	0.65
2H₆	0.90		<i>a</i>		>20	
H₄1	0.10	0.6	0.90	1.9	0.11	0.31
H₄2	0.84	1.0	0.75	0	1.1	>20
H₄3	<i>a</i>	0.9	0.38	1.6	<0.1	0.56
2H₄	0.69	0.8	0.31	1.3	2.2	0.6
L₆1	0.68	4.3	0.56	0.4	1.2	11
L₆2	0.39	3.1	0.32	0.2	1.2	16
L₆3	0.07	0	<i>a</i>	0	>10	
SS1	0.40		<i>a</i>		>20	

^a Peaks too small for accurate integration. Product ratio reported as lower or upper bound.