Photosensitised pyrimidine dimerisation in DNA

M. Consuelo Cuquerella,[a] Virginie Lhiaubet-Vallet,[a] Francisco Bosca[a] and Miguel A. Miranda*[a]

Triplet-mediated pyrimidine (Pyr) dimerisation is a key process in photochemical damage to DNA. It may occur in the presence of a photosensitiser, provided that a number of requirements are fulfilled, such as favourable intersystem crossing quantum yield and high triplet energy. The attention has been mainly focused on cyclobutane pyrimidine dimers, as they are by far the most relevant Pyr photoproducts obtained by sensitisation. The present perspective deals with the involved chemistry, not only in DNA but also in its simple building blocks. It also includes the photophysical characterisation of the Pyr triplet excited states, as well as a brief discussion of the theoretical aspects.

1. Introduction

Ultraviolet solar radiation reaching the Earth's surface comprises wavelengths ranging from 290 to 320 nm (UVB) and 320 to 400 nm (UVA). Both UVB and UVA radiations have been demonstrated to induce mutations in DNA that are in the origin of skin cancer. This is a public health problem, aggravated by the increasing use of tanning sunbeds by the general public. Tanning lamps are intended to produce UVA, but they also emit marginally in the UVB. Recently (July 2009) the International Agency for Research of Cancer (IARC) has declared these devices as "carcinogenic to humans", since they have been proven to increase the risk of skin cancer by 75% when used by people under 30 years old.[1, 2]

Although, in principle, longer-wavelength light is less dangerous, it has to be taken into account that defense mechanisms of the human skin towards their deleterious effects are less effective against UVA induced damage.[3] Actually, a number of reports have appeared on the promutagenic character of UVA radiation. Thus, studies performed on animals (opossum,[4] fish,[5] mice[6]) suggest that it provokes the formation of papillomas, squamous cell carcinomas (SCC) and melanomas; however, the role played by UVA-mediated oxidative damage to DNA in melanoma induction, using xiphophorus fishes as model, has been recently questioned.[5, 7]

While UVB is efficiently absorbed by the nucleobases, causing direct photoreactions of DNA, UVA-induced damage is commonly the result of photosensitisation. Thus, modifications in DNA may occur after light absorption by endogenous or exogenous chromophores present in drugs, cosmetic agents, metabolites, etc.

In this context, UVA-photocarcinogenesis has been mostly related to oxidative stress in early studies. Singlet oxygen production and to a lesser extent hydroxyl radical may be involved in the oxidation of guanine (the nucleobase with the lowest redox potential), giving rise to 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dGuo). However, cyclobutane pyrimidine dimers (CPDs) may also arise from UVA irradiation, and their formation yield is even larger than that of 8-oxo-dGuo in human skin.[3, 8] This has been assessed by exposure of healthy human volunteers to UVA light and subsequent analysis of CPDs in their urine or skin.[9, 10]

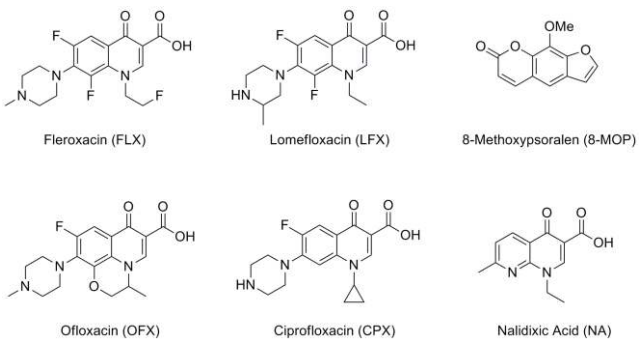


Fig.1 Exogenous photosensitisers acting as photocarcinogens

While the promutagenic character of UVA light is established, the mechanism responsible for UVA photoinduced CPDs formation is a matter of discussion. In particular the possibility of direct UVA-photoinduced damage to DNA, in addition to photosensitisation by endogenous or exogenous agents, is still controversial.[3, 8, 11-17] It has been claimed that CPDs may be formed after UVA irradiation of isolated or cellular DNA in the absence of a photosensitiser. However DNA hardly absorbs UVA, as required for a molecule to react (first law of photochemistry, Grotthus-Draper law). As an alternative to direct DNA excitation, the presence of unknown chromophores or the insufficient purity of the UVA sources used in the experiments has been considered. Moreover, cellular DNA irradiations produce less CPDs in comparison with isolated DNA. As it is difficult to estimate the amount of light absorbed by DNA under these conditions, a contribution by endogenous photosensitisers (*i. e.* porphyrins, flavins, steroids, quinones) cannot be safely ruled out.

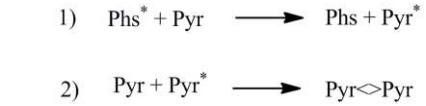
The aim of the present perspective article is to present the case of UVA-photosensitised damage to DNA, with special emphasis on the molecular mechanisms involved in the formation of CPD lesions. A better understanding of these processes should contribute to minimise the photobiological risk.

Among the exogenous agents reported to be photogenotoxic, phototumorigenic and photocarcinogenic *in vivo*, psoralens (used in the PUVA treatment of psoriasis)[18-21] and more recently fluoroquinolones (FQs), have received special attention (Fig. 1). The latter are widely used, broad spectrum antibacterial drugs. They are known to induce UVA-mediated oxidatively damaged DNA,[22-25] and their phototumorigenic potential has been proven in mice.[26-29] Irradiation of albino Swiss and skh-1 hairless mice with UVA light, varying the time of exposure and the drug doses, has established that fleroxacin (FLX) and lomefloxacin (LFX) are more potent phototumorigenic agents than 8-methoxypsoralen (8-MOP). Development of SCCs after the intake of FLX or LFX, together with other lesions such as benign papillomas, solar keratoses or kerato-acanthomas has also been observed in the rodents. Likewise, ofloxacin (OFX), ciprofloxacin (CPX) and the related compound nalidixic acid (NA) have also been found to enhance the development of skin tumours.

In vivo studies on *xeroderma pigmentosum* mice have also revealed LFX as a photocarcinogenic agent. These mice present an inefficient nucleoside excision repair activity for the enzymatic removal of CPDs[29] while conserving the capability to repair oxidatively damaged DNA. Exposure of mice to low UVA doses insufficient to provoke severe phototoxic reactions, leads to a large number of SCCs after only 5 weeks; by contrast, control animals require 23 weeks to show similar effects.

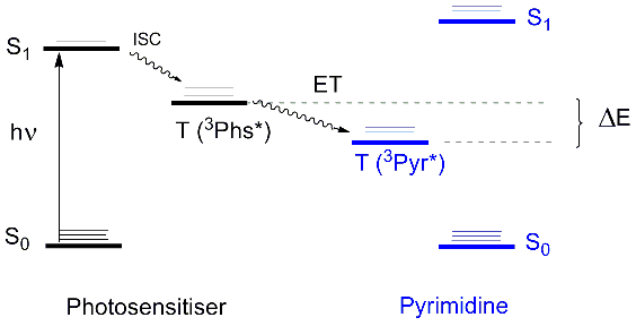
2. Photosensitised formation of cyclobutane pyrimidine dimers

Photosensitised CPDs formation takes place through a formal [2+2] cycloaddition between the C5-C6 double bonds of two pyrimidines (Pyr). Thus, a photosensitiser (Phs) is excited upon light absorption and then transfers its energy to a pyrimidine base, giving rise to thymine or cytosine excited states (Thy^* or Cyt^*). These states are able to react with ground state Thy or Cyt leading to the final products (Schemes 1 and 2).



Scheme 1 Key processes involved in photosensitised Pyr dimerisation

In general, sensitised photocycloadditions are known to proceed through a triplet-triplet energy transfer (TTET) process. As a consequence, a number of requirements should be fulfilled by the Phs of choice: i) to absorb light at longer wavelengths than Pyr, thus allowing for selective excitation, ii) to have a triplet energy above that of Pyr, as requested for thermodynamically favoured process (Scheme 2), iii) to be



Scheme 2 Interconversion between the excited states involved in photosensitised Pyr dimerisation

chemically inert under the reaction conditions, avoiding formation of byproducts and consumption of the Phs, iv) to have a good intersystem crossing quantum yield (ϕ_{ISC}) and a long triplet lifetime (τ_T), in order to increase the probability of energy transfer to an acceptor, and v) to be close enough to the Pyr unit, thereby facilitating collision.

2.1 Efficiency of photosensitised pyrimidine dimerisation

In a TTET process, the energy transfer rate constant (k_{ET}) between the Phs (donor) and the Pyr (acceptor) depends on the energy gap (ΔE), as shown by Sandros' equation:

$$k_{ET} = k_D \frac{1}{\left(e^{-\Delta E/RT} + 1\right)} \quad (1)$$

where k_D is the diffusion rate constant in liquid solutions.[30]

In this context, a favourable value of ΔE is directly related to CPDs formation and therefore to the phototumorigenic capability of endo- or exogenous agents acting as Phs. Thus, it is of paramount importance to establish Pyr triplet excited state energies ($^3Thy^*$, $^3Cyt^*$) to anticipate the potential of a compound to act as Phs. Consequently, an effort has been made in this sense, with special attention to ^3Thy in DNA, where π-stacking and base pairing can have a marked influence on the triplet excited state properties.

In addition to ΔE and temperature[18] (as inferred from eq. 1), photoproducts formation and distribution are influenced by parameters such as the nature of the lowest lying triplet state of the Phs,[31] the solvent,[32] the concentration of Pyr used, etc.

The importance of Pyr concentration deserves a special comment. According to Scheme 1, ground state Pyr quenches both $^3Phs^*$ and $^3Pyr^*$, so the reaction rates should increase with increasing Pyr concentration. In practical terms, photodimerisation quantum yields (ϕ_D) are reproducible only upon complete quenching of the involved triplet excited states. Accordingly, only a limited number of ϕ_D values are available (Table 1), due to the experimental difficulties to cover all the above requirements; they range between 10^{-2} and 10^{-5}. The values are consistent with the upper limit established by measurements performed using acetonitrile (0.02), where formation of cyclobutane thymine dimers (Thy<>Thy) is assumed to occur almost exclusively through the triplet excited state.[33]

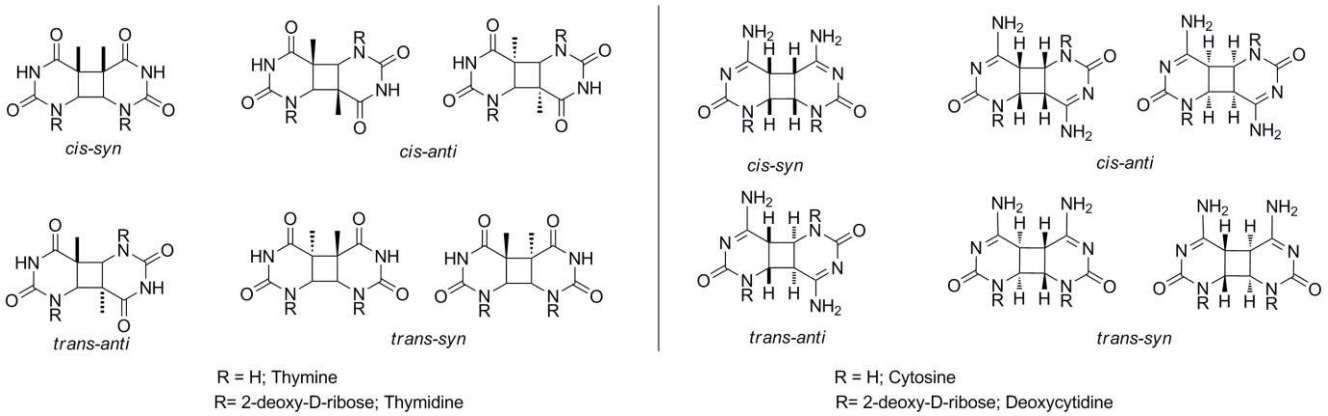


Fig. 2 Structures of all possible homodimers formed after UVA-photosensitised irradiation of Pyr

2.2 Pyrimidine photoproducts in DNA building blocks

In principle, Thy<>Thy, Cyt<>Thy, and Cyt<>Cyt dimers can be formed by photosensitisation of solutions containing the appropriate monomers. Different regio-and diastereoisomers may be obtained in solution (see Fig. 2).[34] It is worth noting that the *cis-anti* and *trans-syn* isomers exist as enantiomeric pairs in Thy and Cyt and as diastereomeric pairs in thymidine (Thd) and 2-deoxycytidine (dCyd). Thus a total of 6 isolable diastereomeric homodimers can be obtained from the nucleosides, as compared with 4 in the case of the free bases.[34, 35]

Although crossed cycloadditions are, in principle, possible to form Thy<>Cyt heterodimers, photosensitised formation of these CPDs has not been described. In this context, Thy dimers have received special attention, since they are the most abundant dimers formed in DNA.[6, 8, 15, 36]

Table 1 Quantum yields of photosensitised pyrimidine dimerisation.

Substrate	Concentration	Photosensitiser	ϕ_D
Thy	1 x 10^{-2} M[a]	Acetone[37]	0.0042
		Acetophenone[37]	0.0016
		BP[37]	0.011
		PABA[38]	0.0007
DMT[c]	0.1 M[b]	BP[39]	0.023
	0.05 M[b]		0.017
	0.1 M[b]	Acetophenone[39]	0.061
	0.2 M[b]		0.031
Supercoiled DNA	18.85 μM in base pairs	Tiaprofenic acid[40]	0.00001
		Ketoprofen[40]	0.0002
		Acetophenone[40]	0.006
Phage T4	-	Cationic acetophenone[41]	0.03

[a] In aqueous solution, [b]in ethyl acetate, toluene, methanol or acetonitrile solutions, [c]BP, benzophenone; PABA, para-aminobenzoic acid; DMT, dimethylthymine.

2.2.1 Thymine and thymidine photosensitisation

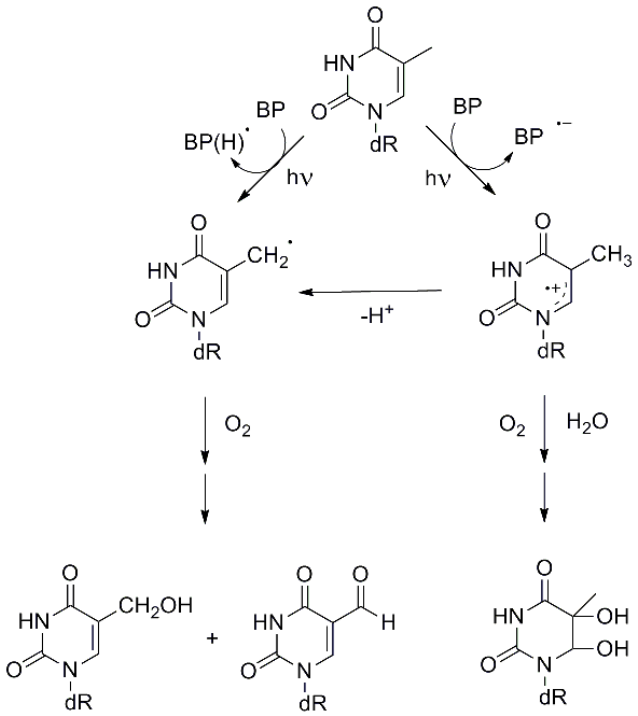
There are a limited number of compounds, including non-steroidal anti-inflammatory drugs (NSAIDS), cosmetic agents like *para*-aminobenzoic acid (PABA), ketones or pyridopsoralens (PyPs), able to photoinduce CPDs formation in free nucleobases or nucleosides; among them PyPs and ketones are very illustrative examples (Fig. 3).

When pyridopsoralens with different triplet energies (E_T), namely, pyrido[3,4-c]psoralen (H-PyPs, E_T = 290.4 kJ/mol), 7-methylpyrido[3,4-c]psoralen (MePyPs, E_T = 288.5 kJ/mol) and 7-methylpyrido[4,3-c]psoralen (2N-MePyPs, E_T = 281.7 kJ/mol) are irradiated in thin films in the presence of Thy, Thy<>Thy dimers are obtained with the *cis-syn* isomer as the most abundant one.[18] Products yields correlate with the PyPs triplet energies, as expected from Sandros' equation. Furthermore, parallel irradiations with the related compounds 5-methoxypsoralen (5-MOP, E_T = 268.2 kJ/mol) and 8-MOP (E_T = 260.5 kJ/mol) show very little if any photosensitised dimer formation.

A clear example of the temperature effect is provided by an experiment performed with H-PyPs. Photoinduced Thy<>Thy formation at 77K is two orders of magnitude less efficient than at 300K. Photosensitisers with E_T lower than that of ^{3}Pyr*[18, 42, 43] can still work upon thermal population of the ^{3}Phs* upper vibrational states. Even if formed in low yields, the resulting CPDs would be of biological significance.[18, 31]

In this context, distribution of the photodimers mixture can be influenced by the polarity of the solvent. Thus, benzophenone (BP) photosensitisation of 1,3-dimethylthymine (DMT) leads to the *cis-syn* isomer as major photoproduct in polar solvents (~ 72%, CH_3CN and CH_3OH) while in non-polar solvents the *cis-anti* one predominates (~50 %, benzene).[32, 39]

Ketones have often been used to photosensitise CPDs formation taking advantage of their high ϕ_{ISC} (nearly 1).[32, 37, 44-49] Irradiation of aqueous solutions of Thy (or Thd) in the presence of acetone, propiophenone, acetophenone or benzophenone gives rise to a mixture of isomers (Fig. 2), with certain prevalence of the *trans-anti* diastereomers.[44, 46-48]



Scheme 3 Mechanistic pathways involved in the photosensitised oxidation of thymidine by benzophenone

Interestingly, ketones can also mediate oxidation of Pyr bases given their ability to participate in hydrogen abstraction or electron transfer processes[50] (Scheme 3). Nonetheless, in the case of dinucleotides such as TpT[48] energy transfer prevails (*ca.* 94 %) over BP-photosensitised oxidation.

2.2.2 Cytosine and 2-deoxycytidine photosensitisation

Although early attempts to photosensitise Cyt<>Cyt formation with BP, acetone or acetophenone were unsuccessful,[37] later work has reported on three dimers of Cyt/dCyd in aqueous acetone (*trans-anti*, *cis-syn* and *cis-anti*, Fig. 2).[35] This type of photoproducts have not attracted special attention, since their photosensitised formation in DNA occurs with comparatively low yields (see below); however Cyt containing CPDs are biologically relevant due to their high mutagenic potential. Besides, Cyt dimers deaminate easily when formed, giving rise to complex mixtures of dimers with random combination of Cyt and uracil (Ura) units. Interestingly, nucleoside deamination has been found to be six times faster than that of the nucleotide.

2.2.3 Byproducts of the photoreaction of triplet sensitisers with pyrimidines

As stated above, a Phs must be chemically inert. Otherwise, secondary reactions can occur giving rise to misleading results, such as formation of byproducts and overestimation of the photoreaction quantum yields. This is the case of PyPs and ketones (Fig. 4).

For example, PyPs possess a double bond in their furan moiety prone to react through a [2+2] cross-photocycloaddition with the C5-C6 double bond of Pyr. Actually, when CPDs formation is photosensitised by PyPs, this type of photoaddition is indeed observed.[19] Likewise in the acetone photosensitised reaction of Thd an acetonyl derivative has been isolated as a side product.[34]

Furthermore, carbonyl compounds may in principle react with alkenes to form oxetane derivatives through a [2+2] photocycloaddition (Paterno-Büchi reaction). This process is favoured when i) the triplet energy of the alkene is comparable to (or higher than) that of the carbonyl compound and ii) the lowest lying carbonyl triplet state is of $n\pi^*$ nature. As a consequence, ketones with relatively low triplet energy may lead to oxetane derivatives of Pyr, in addition to CPDs.[51-53] This is the case of BP:[31,44] its triplet excited state, which presents a $n\pi^*$ configuration, has an E_T level below that of acetone or acetophenone. As a matter of fact, a Paterno-Büchi reaction giving rise to oxetanes is favoured *versus* TTET (Scheme 4). Only when Thd is present at high concentrations CPDs are obtained.[31, 50] Similar observations have been reported for NSAIDs containing the BP chromophore, such as ketoprofen (KP) and its derivatives.[51, 52, 54, 55] Similarly, a photocycloaddition product identified as an oxetane has been described after irradiation of cytosine in acetone-aqueous (1:1) solutions.[53]

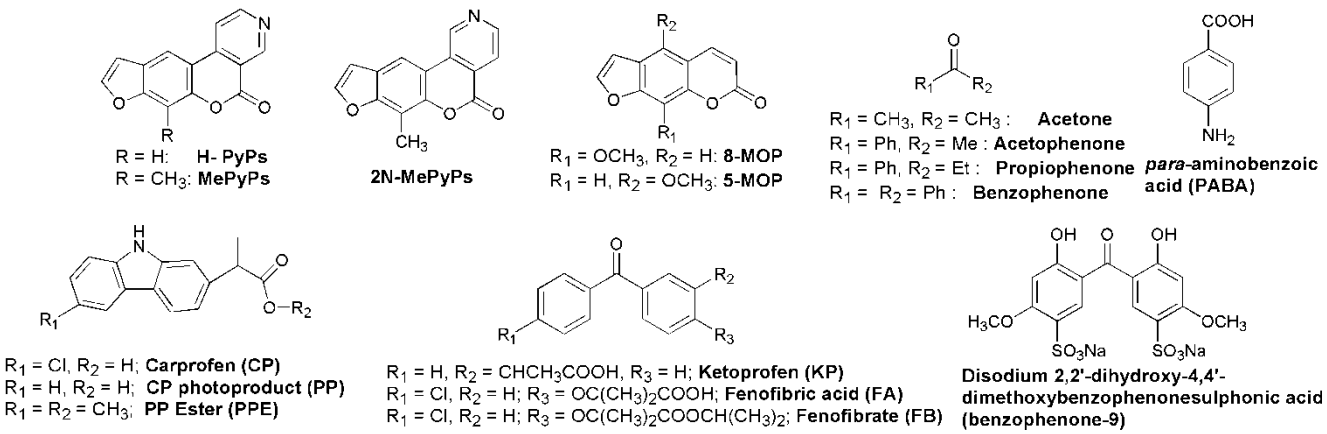
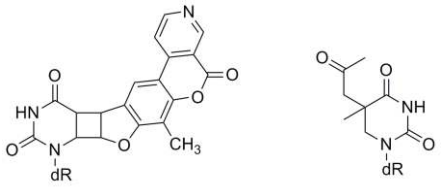
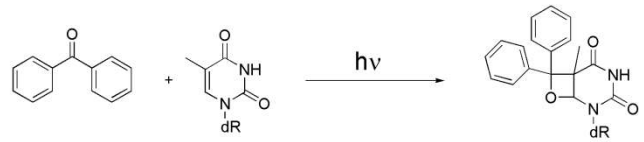


Fig. 3 Photosensitisers for Pyr dimerisation



MePyPs-Thd photoadduct 5-acetonyl-5,6-dihydrothymidine

Fig. 4 Structures of byproducts obtained upon photosensitisation of Thd with PyPs (left) or acetone (right).



Benzophenone (BP) Thymidine (Thd) BP-Thd oxetane

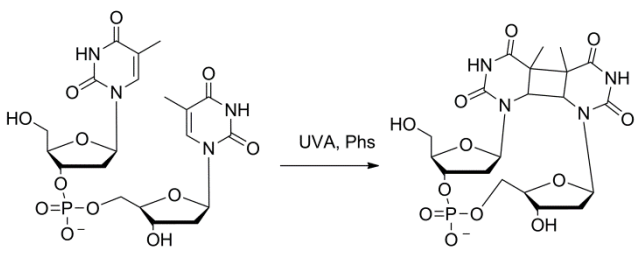
Scheme 4 Paterno-Büchi photoreaction between BP and Thd.

2.3 Oligonucleotides photosensitisation

Photosensitised CPDs formation has been observed in oligonucleotides and single stranded DNA (ss-DNA). Here, Pyr dimerisation occurs through adjacent Pyr on the same strand, inducing a distortion in the structure. Among the possible cyclobutane dimers, 5'-Thy<>Thy-3', 5'-Cyt<>Thy-3' and 5'-Thy<>Cyt-3' are preferentially promoted. However, 5'-Cyt<>Cyt-3' may be obtained in lower yields.[6] Since Cyt is the DNA base with the highest triplet energy, a reduced number of photosensitisers can be involved in a thermodynamically favourable TTET. Furthermore, if a fraction of Cyt reaches the triplet excited state, efficient deactivation by energy transfer to the other bases should be expected.[36]

In addition, Thy<>Thy predominate over Thy<>Cyt dimers in oligonucleotides and DNA.[56] For instance, acetophenone-mediated photodimerisation of thymidylyl-(3'-5')-thymidine (TpT, Scheme 5) occurs five to six times faster than that of thymidylyl-(3'-5')-deoxycytidine (TpdC).[57] The energy gap values in terms of Sandros' equation (eq. 1), together with the relative reactivity of ^{3}Thy towards Cyt or Thy, would explain the different photodimerisation rates.

Orientation restrictions imposed by the sugar-phosphodiester backbone prevent formation of the *anti* forms and favour the *cis-syn* arrangement;[19, 35, 48, 55-59] nonetheless, *trans-syn* dimers are also observed in ss-DNA or



TpT TpT dimers

Scheme 5 Photosensitised thymidylyl-(3'-5')-thymidine (TpT) dimerisation

oligonucleotides owing to their flexible structure.[56, 60] This is the case of dCpT, TpdC and TpT, which produce mainly *cis-syn* and *trans-syn* diastereomers[48, 61] in proportions that may range from 7:1 (TpT) to 3:1(dCpT) or 1:1 (TpdC). Similar results are obtained when two Thy units are kept in close proximity through a polymethylene linker.[62] The largest Thy<>Thy yield is obtained in the case of Thy-$(CH_2)_3$-Thy, likely because the angle between the two Thy approaches that in DNA. Only the intramolecular photodimerisation of some *N*-acetylated dinucleotides gives rise exclusively to the *trans-syn* configuration, presumably due to steric hindrance.[63]

The influence of the Thy-Thy distance has also been evaluated by comparing the reaction rates and Thy<>Thy formation yields of poly(T) and depurinated poly(dA-T). In both cases, Thy<>Thy dimers are formed, albeit the reaction rate is slowed down in the latter due to the poorer π-stacking and longer base-to-base distance. Conformation has a pronounced effect on CPDs formation by governing the extent of stacking between the bases. This is further supported by the fact that dimerisation efficiency is reduced after denaturation by addition of a suitable solvent like ethanol.[36, 64, 65]

The outcome of dimerisation does not only depend on the conformation but also on the nucleobases sequence.[66] The frequency of CPDs lesions increases when a Pyr is located in the 5' side of two consecutive Thy.[56, 67-70] Studies performed by means of ^{32}P radiolabelling and subsequent electrophoresis combined with specific DNA repair enzymes, have shown that photosensitised Pyr<>Pyr formation in a 25-mer 5'-TGA GCG TTA GTT TAA GTC GGC TATC-3' by ketonic drugs occurs more frequently in TTT fragments.

2.4 DNA photosensitisation

While UVA-photosensitisation of DNA gives rise exclusively to CPDs, direct UVB irradiation also produces pyrimidine (6-4) pyrimidone photoproducts.[16] This strongly suggests the involvement of two different mechanisms.

As in oligonucleotides, DNA photosensitisation produces Thy<>Thy, 5'-Cyt<>Thy-3' and 5'-Thy<>Cyt-3', together with small amounts of Cyt<>Cyt in adjacent pyrimidines on the same strand, with an overwhelming predominance of *cis-syn* Thy<>Thy. Analysis of these lesions is often performed by radiolabelling and subsequent electrophoresis.[25, 71, 72] Single cell electrophoresis (comet assay) has been successfully used to reveal cellular DNA damage, as fragmented DNA moves faster through the agarose gel, forming a tail.[73, 74] Both methods can be combined with specific repair enzymes to reveal the type of damage produced.

Relative Pyr<>Pyr formation yields are listed in Table 2. For example, irradiation of DNA using BP or acetophenone as Phs does not produce detectable amounts of Cyt<>Cyt. Only acetone photosensitisation leads to Cyt homodimers,[8, 56, 75, 76] presumably due to the higher E_T. In general, 5'-Cyt<>Thy-3' and 5'-Thy<>Cyt-3' photosensitisation is inefficient as compared to Thy<>Thy, although in ct-DNA and coliphage M13 considerable amounts of heterodimers are obtained. For comparison, in direct DNA photolysis the relative formation yields are 1:0.8:0.2 (TT:CT:CC).[36]

Table 2 Relative Pyr<>Pyr formation yields after UVA irradiation of different types of DNA in the presence of ketones

	BP[a]	Acetophenone[a,b,c,d,e]					Acetone[e,f]	
	a	a	b	c	d	e	e	f
Thy<>Thy	0.2	1	1	1	1	0.65	1	1
Thy<>Cyt	0.046	0.24	0.05	0.19	0.03	*n.d	0.12	0.20
Cyt<>Thy	0.05	0.23						0.36
Cyt<>Cyt	*n.d.	*n.d.	*n.d.	*n.d.	<0.003	*n.d.	0.05	0.08

[a]Calf-thymus DNA,[8] [b]native DNA,[64] [c]denatured DNA,[64] [d]*E. coli* DNA,[41] [e] Phage T7[77] and [f]Coliphage M13 mp2,[56] *n.d. = Not determined. For each experiment, the amount of Thy<>Thy has been set as the unity for comparison.

Formation of CPDs in isolated and cellular DNA can be photosensitised not only by ketones,[58, 76] but also by PyPs,[19, 78] NSAIDs,[40, 54, 68, 79] FQs,[23, 25, 29, 71-74, 80, 81] amino acids and derivatives[59, 82] or cosmetic agents[83, 84] (Fig. 5). Studies performed on Thy dimerisation by the FQ family have played a key role in determining the triplet energy of Thy in DNA at *ca* 267 kJ/mol. Dimers formation is mediated by ENX and NFX, while it is not by the N(4')-acetyl NFX derivative (ANFX) or OFX.[23, 25, 71, 72] Interestingly, for ENX, LFX and NFX the efficiency of CPDs formation has been found to be different in isolated and cellular DNA.

As in oligonucleotides, Thy<>Thy formation in DNA is sequence-dependent.[19, 56, 69, 78] Pyridopsoralens H-PyPs, MePyPs and 2N-MePyPs react specially at TTTTA and TTAAT fragments, provoking 40% and 55% of Thy<>Thy photolesions, respectively. Moreover, CPDs formation is not detected in a GC environment or at CC sites.

In addition to neighbouring effects, sequence dependence may be the result of selective formation of Phs-DNA complexes in specific DNA locations. Thus, complexation to DNA can place the Phs and the Pyr units in close proximity favouring TTET processes. As an example, 4',5'-dihydro-7-methylpyrido[3,4-c]psoralen, a modified PyPs, binds to DNA close to 5'-TA-3' sites.[78] If the binding is disrupted (*i.e.* by varying the ionic strength), Thy dimers formation is negligible.[82] An additional example is provided by two cationic derivatives: β-dimethylaminopropiophenone hydrochloride and *N*-(*m*-acetylbenzyl)-*N*-(2-aminoethyl) ammonium dichloride. The charged Phs are brought close to DNA by ionic interactions, which is reflected in an increased photosensitisation capability with respect to acetophenone.[41]

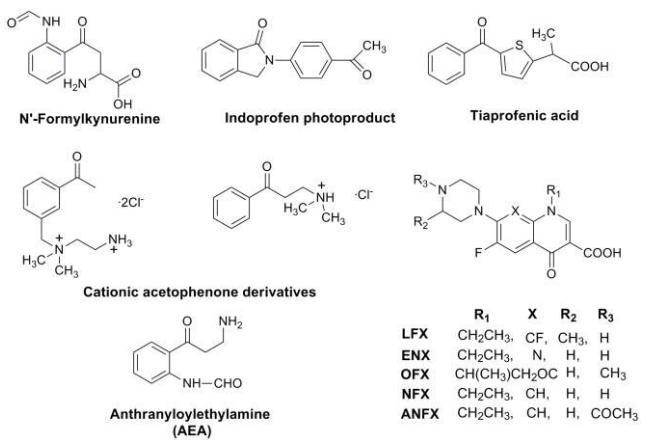


Fig. 5 Photosensitisers for Pyr<>Pyr formation in DNA

3. Spore photoproducts

Another interesting dimeric pyrimidine lesion corresponds to the most abundant UV photoproduct in bacterial spores.[85, 86] Indeed, 5-thyminyl-5,6-dihydrothymine adduct, the so called spore photoproduct (Fig. 6, SP), is formally obtained by linking the allylic carbon to the C5 position of a neighbouring Thy, with saturation of the C5-C6 double bond. Four isomeric forms of SP may be formed within DNA since a chiral center is generated at C5a carbon. Furthermore, the allylic carbon can be linked to adjacent thymines located

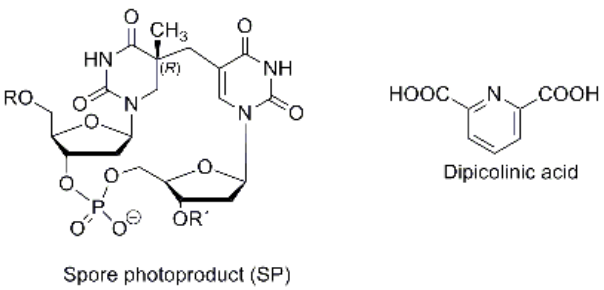
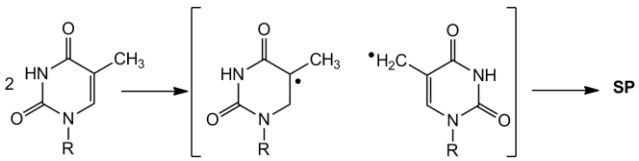


Fig. 6 Structures of SP photoproduct and of dipicolinic acid

either at the 3´- or at the 5´-end. Interestingly, DNA double helix structure induces a highly stereospecific formation of SP photoproducts (Fig. 6);[87] their stucture has been recently assigned by 2D NMR studies combined with DFT calculations. Thus, the natural SP results from addition of the thymine methyl group located on the 3´-end to the thymine C5 carbon located in the 5´-end, giving rise to a new chiral center with *R* absolute configuration.[87] Moreover, SP is only obtained as a Thy homodimer and has been detected both as intrastrand and interstrand lesion.[88, 89]

Indeed, SP is a quite peculiar bipyrimidine photoproduct, whose formation has been related to three important factors:[85, 89, 90] i) the low hydration level in spore core, ii) the binding ofα/β type small, acid soluble protein (SASP), which converts DNA from B-like to A-like conformation, and iii) the presence of dipicolinic acid (pyridine-2,6-dicarboxylic acid DPA, Fig. 6) in the spore core (up to 10% of dry weight). The



Scheme 6 Mechanism postulated for SP formation.

photosensitising properties of this endogenous compound have been first proposed on the basis of the decrease of SP formation yield in spore strains lacking DPA.[88, 91] This hypothesis has been further supported by studies in less complex media like isolated DNA, TpT or Thd.[49, 87-89, 92, 93] In this context, UVC irradiation of DNA dry films in the presence of DPA has revealed the increase of SP, Thy<>Thy, and to a lesser Thy<>Cyt and Cyt<>Thy relative yields,[85, 88, 91] whereas Cyt<>Cyt and (6-4) photoproducts yields remain almost unchanged. These data are in agreement with the role of DPA as triplet photosensitiser, acting as donor in TTET processes. Further pieces of evidence supporting the feasibility of such a process have been provided by the results of UVA-irradiation of Thd dry films in the presence of BP or three pyridopsoralen derivatives (H-PyPs, MePyPs and 2N-MePyPS, Fig. 3).[19, 49] In these experiments, the six diastereoisomers of Thd<>Thd and the 5*R**/5*S** diastereoisomers of SP have been detected; their yields have been shown to depend on the nature of the photosensitiser.[19] As expected for triplet energy donors, their efficiency can be related to their excited state level, with H-PyPs being the most efficient, followed by MePyPs and 2N-MePyPs. Accordingly, no formation of Thd<>Thd and SP has been observed for the donors with lower triplet energy like 3-carbethoxypsoralen, 5-methoxypsoralen and 8-MOP.[19]

Finally, it is noteworthy that in spite of the interesting SP photochemistry, the involved mechanism has not been investigated.[85, 94] The reaction may occur by coupling of the 5-thyminyl /5,6-dihydrothymin-5-yl radical pair generated as a result of H-abstraction from a ground state by a triplet excited Thy (Scheme 6). Alternatively, a concerted mechanism involving the methyl group of one Thy and the double bond of the second Thy has been proposed. Either the shorter lifetime of radical pairs (as compared with the free radicals generated by radiation)[95, 96] or the concerted nature of the process would account for the observed stereoselectivity.

4. Theoretical calculations

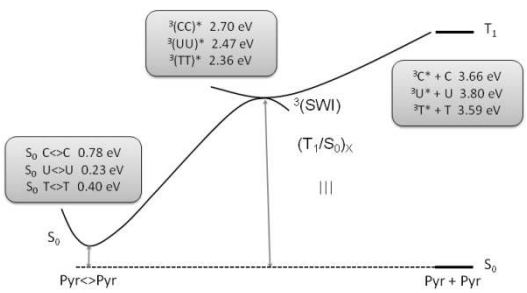
In spite of their importance in DNA damage formation, the Pyr triplet excited states are only now starting to be analysed by theoretical studies.[97-108] First principles calculation methods converge on the ππ* nature of the lowest triplet state but not on their energy values, which have been reported over a large range (Table 3) depending on the method used for calculation and geometry optimisation. So far, the highest level of approach has been CASPT2 based on CASSCF wave functions. However, because of the difficulties to apply this methodology for large molecules, a number of calculations have been performed at the density functional theory (DFT) level or by using the coupled cluster (CC) model.

In this context, the vertical excitation energy for Thy at the ground state geometry is situated between 336.7 and 382.1 kJ/mol (3.49 eV and 3.96 eV), while adiabatic excitation energy values range from 272.1 to 304.9 kJ/mol (from 2.82 to 3.16 eV). Similar discrepancies have been reported for Cyt and Ura (Table 3). Nevertheless, the general trend extracted from the calculated values is in agreement with the experimental data, *i.e.* the nucleobase with the lowest triplet excited state energy is Thy, while the highest triplet manifold corresponds to Cyt. Recently, the energetic gap between ^{3}Thy* and ^{3}Ura* has been rationalised in terms of the influence of C5-methylation. This substitution induces an up-shift of the π HOMO while the acceptor π* LUMO is almost unaffected, thus resulting in a red shifted π→π* transition.[100]

Table 3 Vertical and adiabatic π→π* (singlet→triplet) transition energy calculated for isolated Thy, Cyt and Ura and for their excimers (Pyr-Pyr). Values are given in kJ/mol and in eV in parentheses.

	Vertical	Adiabatic
Thy	336.7-337.7 (3.49-3.50)[a] [97] 366.6-377.3 (3.80-3.91)[b] [97] 358.9-372.4 (3.72-3.86)[c] [98] 341.6 (3.54)[a] [98] 373.4 (3.87)[c] [100] 376.3 (3.90)[c] [100] *hyd,* 382.1 (3.96)[c] [100] *aq* 346.4 (3.59)[d] [99] 368.6 (3.82)[c] [107]	272.1-275.0 (2.82-2.85)[a] [97] 288.5-296.2 (2.99-3.07)[b] [97] 299.1-304.9 (3.10-3.16)[c] [98] 285.6 (2.96)[a] [98] 296.2 (3.07)[c] [100] 276.9 (2.87)[d] [99]
Thy-Thy		274.6-275.5 (2.84-2.85)[a] [102] 227.7 (2.36)[d] [103]
Cyt	347.3- 350.2 (3.60-3.63)[a] [97] 361.8-383 (3.75-3.97)[b] [97] 352.2 (3.65)[d] [101] 330.0-352.2 (3.42-3.65)[a] [105] 361.8-383.0 (3.75-3.97)[b] [105] 340.6 (3.53)[d] [106] 374.4 (3.88)[c] [107]	293.3-298.1 (3.04-3.09)[a] [97] 302.0-311.6 (3.13-3.23)[b] [97] 293.3 (3.04)[d] [101] 284.6-298.1 (2.95-3.09)[a] [105] 302.0-311.6 (3.13-3.23)[b] [105] 287.5 (2.98)[d] [106]
Cyt-Cyt		260.5 (2.70)[d] [103]
Ura	370.5-383.0 (3.84-3.97)[c] [98] 367.7 (3.78)[a] [98] 384.0 (3.98)[c] [100] 387.9 (4.02)[c] [100] *hyd* 395.6 (4.10)[c] [100] *aq* 366.6 (3.80)[d] [108] 379.2 (3.93)[c] [107]	284.6-383.0 (2.95-3.97)[a] [97] 287.5-419.7 (2.98-4.35)[b] [97] 312.6-319.4 (3.24-3.31)[c] [98] 302.0 (3.13)[a] [98] 309.7 (3.21)[c] [100] 303.9 (3.15)[d] [108]
Ura-Ura		238.3 (2.47)[d] [103]

Calculated using [a] DFT, [b] CCSD, [c] CC2, [d] CASPT2//CASSCF



Scheme 7 Triplet mediated formation of Pyr<>Pyr. Adapted from Serrano *et al* [103]

Computational studies are generally performed *in vacuo i.e.* without taking into account interaction between the target molecules and solvent. However, it is well established that such interactions may have a marked influence on the excited state energy. In this context, the combined effect of hydration (energies denoted as *hyd*, Table 3) and solvent polarity, used to mimic aqueous environment (denoted *aq* in Table 3) has only revealed a slight destabilisation of the lowest $^3\pi\pi^*$ triplet state of Ura and Thy.[100, 109] Concerning the photochemical reactivity of Pyr, theoretical calculations have mainly focused on the formation of cyclobutane dimers in a concerted process from the singlet excited state.[110-114] However, as stated above, in the case of photosensitised Pyr<>Pyr formation, [2+2] photocycloaddition occurs from the triplet manifold. In spite of its importance, up to now this reaction pathway has only been considered by two research teams.[102-104] In a first approach, calculations at the TD-DFT level have shown that triplet mediated photocycloaddition of Thy proceeds through an initial C6-C6´ bond formation, leading to a biradical intermediate that subsequently crosslinks to the singlet surface, giving rise to Thy<>Thy.[102] More recently, Pyr<>Pyr formation has been computed by means of high level quantum chemical CASPT2//CASSCF calculations.[103, 104] For a better approach to the nucleobase properties in DNA, the triplet minima have been calculated for excimer arrangements formed by the parallel stacking of the bases (Scheme 7). In this context, a stabilisation relative to the isolated bases (denoted ^{3}Pyr*+Pyr in Scheme 7) is observed, giving rise to adiabatic energy values of 2.36, 2.47, 2.70 eV for Thy, Ura, and Cyt, respectively. This triplet excimer connects without any energy barrier with a stepwise intermediate ^{3}(SWI), which exhibits a biradical character. Indeed, a covalent bond is formed between the C6 and C6´ carbons, with the unpaired electrons and spin density located on the two other ethylenic carbons (C5 and C5´). At this stage, the calculated C6-C6´ bond lengths are 1.669, 1.660 and 1.664 Å for Cyt, Ura, and Thy; by contrast, the interatomic C5-C5´ distance is elongated (about 2.8 Å). Finally, ^{3}(SWI) corresponds to a singlet-triplet crossing $(T_1/S_0)_x$ structure leading to Pyr<>Pyr ground state.

Thus, the efficiency of Pyr<>Pyr photosensitisation depends on two factors. The first one is the effectiveness of the TTET process from the ^{3}Phs*, which is related with the triplet state energy of the nucleobase (^{3}Cyt*>^{3}Ura*>^{3}Thy*); this agrees with the experimental predominance of Thy<>Thy dimers *vs.* other Pyr combinations. The second factor deals with the efficiency of the intersystem crossing process toward the ground state of the photoproduct $(T_1/S_0)_x$.

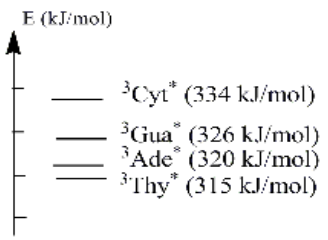


Fig. 7 Energies of DNA bases determined at 77K in ethylene glycol/H_2O[42]

5. Photophysics

5.1 Emission

Thymine and its derivatives exhibit a pH-dependent phosphorescence emission. At 77 K under neutral conditions, no signal is observed for diluted aqueous solutions.[115-117] Conversely, at a pH higher than the pKa of Thy (*ca.* 9.6),[118] the nucleobase in its anionic form emits at 445 nm with a decay time of 0.4-0.5 s.[115, 119, 120] Parallel experiments have reported that at neutral pH and high concentrations (*i. e.* 10^{-3}-10^{-2} M), formation of aggregates gives rise to a phosphorescence emission at 470 nm, with a lifetime of 0.2 s (Table 4).[42, 118] A similar spectrum has been detected by photosensitisation experiments using acetone to populate the Thd monophosphate (TMP) triplet excited state by an energy transfer mechanism.[42, 120] Emission of Cyt and its derivatives has been studied to a lesser extent;[43, 120-125] the obtained data are listed in Table 4.

Thus, as shown in Figure 7, Thy and Cyt are the nucleobases with the lowest and the highest triplet excited state energies, respectively.

An intriguing result is the reported phosphorescence emission of native DNA. Indeed, by considering the higher phosphorescence quantum yield of purines, an overall emission closely related to these bases could be expected. However, the DNA spectrum does not exhibit the well-structured band characteristic of purines, and the obtained quantum yield (Table 4) is 1 order of magnitude lower than that of adenosine monophosphate (AMP) or guanosine monophosphate (GMP). Hence, it has been proposed that DNA emission arises from the triplet level of Thy residues. Accordingly, a more intense phosphorescence has been monitored for DNA with higher adenine (Ade) + Thy contents.[42, 126] Different hypotheses have been postulated to explain the nature of the emissive residues. On the basis of the lack of phosphorescence found for isolated Thy, emission was initially attributed to that of Thy anion formed by transfer of the thymine N3 proton across the Watson-Crick base pairing to the N1 nitrogen of adenine.[115, 119] Nevertheless, this hypothesis has been contradicted by the phosphorescence emission of Thy aggregates, which closely resembles that of DNA. This assignment has been further supported by the Thy-like emission of 1,3-dimethylthymine in the presence of Ade, where the proton transfer is not possible.[118, 127] A similar conclusion has been drawn from single stranded DNA studies.[42] Different explanations have been provided to account for the fact that Thy is the only emitting residue in DNA. The first one is related to its relatively low triplet state energy. It has been reported that, irrespective of the excited chromophore (Ade, Gua, Thy or Cyt), the only emission observed in solutions containing mixtures of the nucleobases is that of ^{3}Thy*.[118, 120, 127] This is consistent with energy transfer from the higher lying triplet excited states of Cyt, Ade or Gua to Thy in solution. Accordingly, a thymine emission has also been obtained for the dinucleotides dApT and TpdA but also for polydAT.[42, 115, 119, 120, 126, 128] A similar deactivation channel towards ^{3}Thy* has been postulated to explain the case of the whole DNA biomacromolecule.[118, 127]

Table 4 Phosphorescence emission properties of pyrimidines, oligonucleotides and DNA at 77K. If not specified the values correspond to ethylene glycol/water glass at neutral pH.

	λ_{max} (nm)	τ (s)	ϕ_{em}
Thy	470[118, 127] 460[a] [129]	0.5[43] 0.075[a] [129]	<0.008[122] 0.006[43] 0.018-0.015[a] [129]
Thd	470[b] [127] 460[a] [129] 450[d] [123]+	0.21[b] [127] ≤0.5[c] [122] 0.6[d] [123]	<0.015[122] 0.006[43] 0.038-0.042[a] [129]
TMP	440[42, 120]	0.3[42, 120] ≤0.4[122]	0.008[43] ≤0.01[122]
dApT (or TpdA)	440[120]		0.021 (0.009)[120]
polydAT	450[126] 448[119]	0.3 [42, 115, 119, 126, 128]	≥0.004[119]
DNA	450[126] 448[119] 450[d] [123]	0.3 [42, 115, 119, 126] 0.3[g] [119] 0.5[d] [123]	0.002 [115, 119] ≤ 0.02[122]
Cyt	430[e] [123]	0.8[c] [122] 0.6[e] [123]	0.006[122]
dCyd	435[b] [127] 430[f,b] [127] 410[d] [123]	0.6[b] [118] 0.66[c] [122] 0.6[d] [123]	0.009[122]
dCMP	410[h] [120, 124]	0.4[122] 0.34[120, 125]	0.015[122] 0.01[120]
dCpdC			0.01[120]
polyC	420[d] [123]	0.7[119] 0.6[122]	0.02[122]

[a] 2-methyltetrahydrofuran, [b] In H_2O pH 7; [c] in CH_3CH_2OH; [d] : 0.25% glucose in 0.1M sodium acetate-H_2O, [e] isopropanol/isopentane, [f] H_2O /propylene glycol 1/1, [g] H_2O, [h] CH_3OH/H_2O 1/9 pH 7.

On the other hand, for the Gua – Cyt base pair, proton transfer at the singlet level can also be in the origin of their lack of emission.[118, 119] Expectedly, DNA structure integrity is an important parameter. Temperature, solvent or pH conditions leading to denaturation of the double helix result in the typical blue shifted, more structured and longer lived phosphorescence emission of purines.[119, 126]

5.2 Laser flash photolysis studies

5.2.1 Intrinsic population of the triplet excited state upon direct excitation of Pyr bases

Time-resolved techniques have been used to study the photophysical properties of singlet and triplet excited states of individual nucleobases, nucleosides, nucleotides and oligonucleotides.[130-142] In this context, it is generally accepted that the mechanisms involved in the generation of CPDs upon direct excitation of Thy are initiated by population of a singlet ππ* state;[138, 139, 141] however, the nature of the subsequent steps still remains a matter of discussion. Recent studies performed on Thd and $(T)_{20}$, using ultrafast time-resolved fluorescence and transient absorption spectroscopy, suggest that the ππ* triplet excited state is a key player in the dimerisation reaction. Thus, similar spectra (with maxima at 350-400 nm) are obtained for both Thd and $(T)_{20}$.[136, 141, 142] They have been safely assigned to the T-T transition, which fully develops within picoseconds. The ISC quantum yield in aqueous solution has been estimated to be in the range 0.01-0.03, with a higher value for $(T)_{20}$ than for Thd. The main difference observed between the nucleoside and the oligonucleotide concerns the triplet lifetimes. Thus, τ_T values in the subnanosecond domain have been determined for $(T)_{20}$; by contrast, ^{3}Thd survives in the ns-μs timescale, with a concentration-dependent lifetime. The role of triplet-mediated CPDs formation in $(T)_{20}$ supports a major contribution of this pathway also in natural DNA, where an extremely short τ_T value would account for the previously reported inefficient quenching of CPDs formation by oxygen. Interestingly, the quantum yield of Pyr<>Pyr photodimerisation in double-stranded genomic DNA is *ca.* 30 times lower than in $(T)_n$ oligonucleotides.[3, 138, 139] Light absorption by non-thymine bases, low frequency of Thy doublets, and conformational restrictions may be in the origin of this effect.

Table 5 shows a selection of key photophysical parameters (ISC quantum yield, as well as rate constants for unimolecular decay and self quenching, k_0), determined for the triplet excited state of the Thy chromophore in the free base and in some derivatives. It also includes the corresponding molar absorption coefficients of the T-T absorption (ε_T), which have been obtained applying an energy transfer method, with retinol as acceptor.[130] The data indicate that formation of the triplet excited states upon direct UV-irradiation occurs actually in all cases. In spite of its biological relevance, the efficiency of this process is low, particularly in aqueous medium.

Table 5 Photophysical properties of the triplet excited state of Thy and its derivatives in different solvents

	Solvent	ε_T ($M^{-1}cm^{-1}$) at 370 nm[141, 143]	ϕ_{ISC} / 10^2 [141, 143]	k_0 (s^{-1}) / 10^5 [136, 143]	k_S ($M^{-1}s^{-1}$) / 10^8 [136, 143]
Thy	CH_3CN	2700	6.0	0.7-2.2	5.3-7.0
	H_2O	3500	0.6	0.2	7.9
Thd	CH_3CN	3600	6.9		
	H_2O	3600	1.4	0.4	1.0-1.9
TMP	CH_3CH_2OH	4000	5.5		2.0
	H_2O	3500	0.8-1.5	0.4	0.2
$(T)_{20}$	H_2O	2700	2.8		100[a]

[a]Rate constant given in s^{-1}

As stated above, triplet quenching by the ground states is associated with Pyr<>Pyr dimerisation. Its rate constant can be determined according to:

$$k_{obs} = k_0 + k_S [S_0] \quad (2)$$

where k_s stands for unimolecular decay and self quenching, respectively. As a matter of fact, the obtained values show that both deactivation pathways can compete, depending on the experimental conditions (solvent, concentration, etc.). It is worth noting that the highest k_S corresponds to the oligonucleotide, as expected for a concentration-independent process. A similar situation can be anticipated for natural DNA.

By contrast with Thy derivatives, the triplet excited states of Cyt, dCyd and dCyd monophosphate (dCMP) have not been observed upon direct photolysis; this can be atributed to the low ISC quantum yields and molar absorption coefficients.[133]

5.2.2 Photosensitised generation of Pyr triplets

A number of time-resolved studies have been performed on photosensitised reactions of Pyr bases, either alone or as substructures of more complex entities (nucleosides, nucleotides, oligonucleotides, etc.). In this context, Pyr dimerisation has been shown to occur via the triplet excited state, using sensitisers such as acetone, acetophenone and propiophenone (Fig. 3).[37, 132-136] Acetone is particularly advantageous over other triplet sensitisers in kinetic studies for several reasons. First, acetone has a very high triplet energy (E_T *ca.* 330 kJ) and hence it can photosensitise both Thy and Cyt bases. Second, its ISC quantum yield is close to the unity, so it is two orders of magnitude higher than those described for Pyr derivatives. Finally, the triplet-triplet absorption band of acetone does not interfere with observation of the growth of Pyr triplet excited states at 400 nm, and does not overlap with their whole transient absorption spectra (see Figure 8).[133, 136]

After excitation of the photosensitiser, a number of processes may occur. The initial step is formation of the Phs first singlet excited state ($^1Phs^*$). At this point, several pathways can compete: fluorescence and internal conversion lead back to Phs, while intersystem crossing affords the triplet excited state ($^3Phs^*$). An ideal photosensitiser should have an ISC quantum yield close to 1.

The Phs triplet energy relative to that of the Pyr derivative is a key point to predict whether energy transfer can proceed. Thus, for irreversible energy transfer, the triplet energy of the donor must be at least 12 kJ/mol higher than that of the acceptor.[135]

In this context, the interaction between a variety of ketone triplets and mononucleotides has been studied as a function of the relative energies of the Phs-nucleotide pair. While ketones with E_T higher than 305 kJ/mol (acetone, acetophenone, propiophenone and 1-indanone) sensitise the generation of a transient absorption corresponding to $^3TMP^*$ in laser flash photolysis (LFP), those with E_T < 305 kJ/mol do not exhibit any triplet sensitisation capability, in spite of the significant quenching experimentally observed ($k_q > 10^8$ M^{-1} s^{-1}).[134]

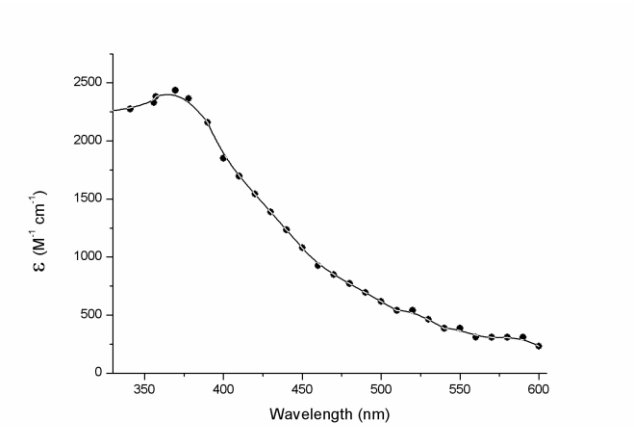


Fig. 8 Transient absorption spectrum of triplet excited of TMP, obtained by energy transfer from acetone in deaerated aqueous solutions.[134]

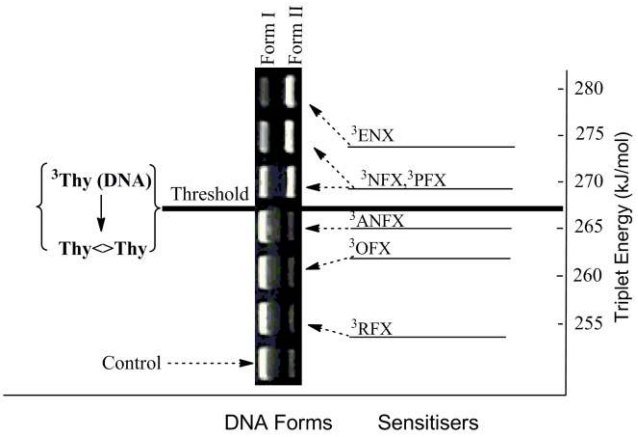


Fig. 9 Triplet energy of photosensitisers and electrophoretic analysis of DNA Form I (supercoiled native form) and Form II (single strand break) obtained from mixtures containing pBR322 and FQs (20 μM) after 15 min of irradiation and subsequent T4 Endo V treatment. Adapted from Lhiaubet-Vallet *et al.*[72]

Hence, the absolute value of TMP triplet energy has been estimated at *ca.* 310 kJ/mol. According to the Sandros' equation, this is consistent with the observation of energy transfer from acetophenone (E_T = 310 kJ/mol), but not from 3-methoxyacetophenone (E_T = 303 kJ/mol).[135]

Benzophenone derivatives, with E_T = 290 kJ/mol, lower than that of TMP, have been shown to photosensitise Thd<>Thd formation at high nucleoside concentrations, in competition with a more favoured Paterno-Büchi photocycloaddition.[31] This explains the efficient quenching (k_q = 5.1 x 10^8 M^{-1} s^{-1}) observed in acetonitrile. Remarkably, LFP studies on (*S*)- and (*R*)- KP have shown a significant enantiodifferentiation in the quenching rate constants by Thd.[55]

In DNA, the photosensitiser triplet energy required for Pyr<>Pyr formation has been progressively shifted from *ca.* 300 kJ/mol (methoxyacetophenones)[134] down to 290 kJ/mol (benzophenone and phthalimidine derivatives)[31, 48, 55, 68] and more recently to 267 kJ/mol (FQs)[71, 72]. A series of fluoroquinolones (FQs), including ENX, pefloxacin (PFX),

NFX, ANFX, OFX and rufloxacin (RFX) have been investigated to determine their potential as photosensitisers for Pyr<>Pyr dimers formation in DNA. At FQ concentrations and light doses insufficient to produce direct single strand breaks, ENX, PFX and NFX are able to produce Pyr<>Pyr dimers in DNA as revealed by enzymatic treatment with T4 endonuclease V. By contrast, ANFX, OFX and RFX are inefficient in this assay (Fig. 9). This information has been combined with the absolute values of the triplet energies of ENX, PFX, NFX, ANFX, OFX and RFX, estimated by means of LFP, using flurbiprofen (FBP), 4-biphenylcarboxylic acid (BPC) and naproxen (NP) as energy acceptors (Table 6).[71, 72]

Table 6 Rate constants of energy transfer from FQs to flurbiprofen (FBP), 4-biphenylcarboxylic acid (BPC) and naproxen (NP) and estimated values of the triplet excited state energies of FQs[72]

	^{3}ENX	^{3}PFX	^{3}NFX	^{3}ANFX	^{3}OFX	^{3}RFX
k_{ET} (FBP) / 10^9 ($M^{-1}s^{-1}$)[a]	0.3	0.09	0.09	< 0.01	< 0.01	< 0.01
ΔE_{FBP-X} (kJ/mol)[b]	+4	+8	+8	> +8	> +8	> +8
k_{ET} (BPC) / 10^9 ($M^{-1}s^{-1}$)[a]	2.3	1.7	1.5	0.9	0.02	< 0.01
ΔE_{BPC-X} (kJ/mol)[b]	< -8	-3	-3	+1	> +8	> +8
k_{ET} (NP) / 10^9 ($M^{-1}s^{-1}$)[a]	2.3	2.2	2.2	2.1	1.8	0.2
ΔE_{NP-X} (kJ/mol)[b]	< -8	< -8	< -8	-7	-4	+6
E_T (kJ/mol)[c]	273	269	269	265	262	253

[a] Quenching rate constants obtained in N_2O-purged medium using 0.1 to 10 mM concentrations of the quenchers. [b]ΔE is the energy difference between the triplet excited states of quenchers and FQs obtained from Sandros´ equation; $k_{ET} = (k_{max} \times e^{-\Delta E/RT}) / (e^{-\Delta E/RT} + 1)$ assuming that $k_{max} = 2.2 \times 10^9$ $M^{-1}s^{-1}$.[c]Estimated values taking into account that E_T of BPC and NP are *ca*. 266 and 259 kJ/mol, respectively

All the results indicate that the threshold E_T value required for a given compound to become a potential DNA photosensitiser *via* Thy<>Thy formation is in the range defined by the triplet energies of NFX and ANFX (265-269 kJ/mol, see Fig. 9).

Moreover, when the Phs E_T is lower than the threshold, triplet quenching by Pyr derivatives can only occur by pathways not involving energy transfer. This is the case for menadione, whose E_T is ca. 243 kJ/mol. The high quenching rate constants (between 1.0 and 2.5 x 10^9 M^{-1} s^{-1}) correspond to electron transfer, generating menadione radical anion and Pyr radical cations (Scheme 3).[144]

As stated above, in the case of Cyt, dCyd and dCMP, the triplet energy is higher than that reported for Thy, Thd and TMP. Therefore their triplet excited states are difficult to detect when both types of Pyr units are present, owing to deactivation *via* base-to-base energy transfer. Nonetheless, the transient absorption spectra have been recorded using acetone as Phs. From this type of experiment, it has been possible to determine the rate constants of unimolecular decay (k_0), as well as those of self quenching (k_s) (Table 7).[133]

Table 7 Kinetic parameters of Cyt and derivatives in aqueous solutions

	k_0 /10^4 s^{-1}	k_S / 10^8 $M^{-1}s^{-1}$	k_{q1} (acetone) /10^9 M^{-1} s^{-1}
Cyt	5.5	4.2	3.8
dCyd	7.6	2.4	4.5
dCMP	9.4	1.8	5.1

6. Summary and Outlook

Triplet excited states play a key role in the dimerisation of pyrimidine bases, not only in photosensitised processes, but also upon direct UV-irradiation. Cyclobutane pyrimidine dimers (CPDs) are by far the most relevant Pyr photoproducts obtained by sensitisation. Spore photoproducts may also be formed through this pathway; however, they are not found in mammals or other higher organisms. By contrast, there is no evidence for photosensitised reactions leading to pyrimidine (6-4) pyrimidone photoproducts. The mechanism of direct UVA-induced CPDs formation is still controversial; as DNA hardly absorbs in this wavelength range, the involvement of endogenous photosensitisers cannot be safely ruled out. In spite of the importance of the triplet pathway in Pyr photodimerisation, further efforts are needed to achieve a better understanding by means of theoretical calculations. Issues such as the lack of pyrimidine (6-4) pyrimidone formation or the special conditions required to obtain spore photoproducts from the triplet manifold should be explained. Finally, the triplet energy of thymine in DNA has been found to be much lower than that of the free base or the nucleoside. As this is a key parameter to anticipate the potential photocarcinogenicity of photosensitisers, it seems interesting to clarify how the various structural features (sequence, π-stacking, base pairing, etc.) modulate its actual value in the different microenvironments of the biomacromolecule.

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Notes and references

[a] *Instituto de Tecnología Química UPV-CSIC, Universidad Politécnica de Valencia, Avda de los Naranjos s/n, 46022 Valencia, Spain Fax: +34 963879444 ; Tel: +34 963877807; E-mail: mmiranda@qim.upv.es*

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