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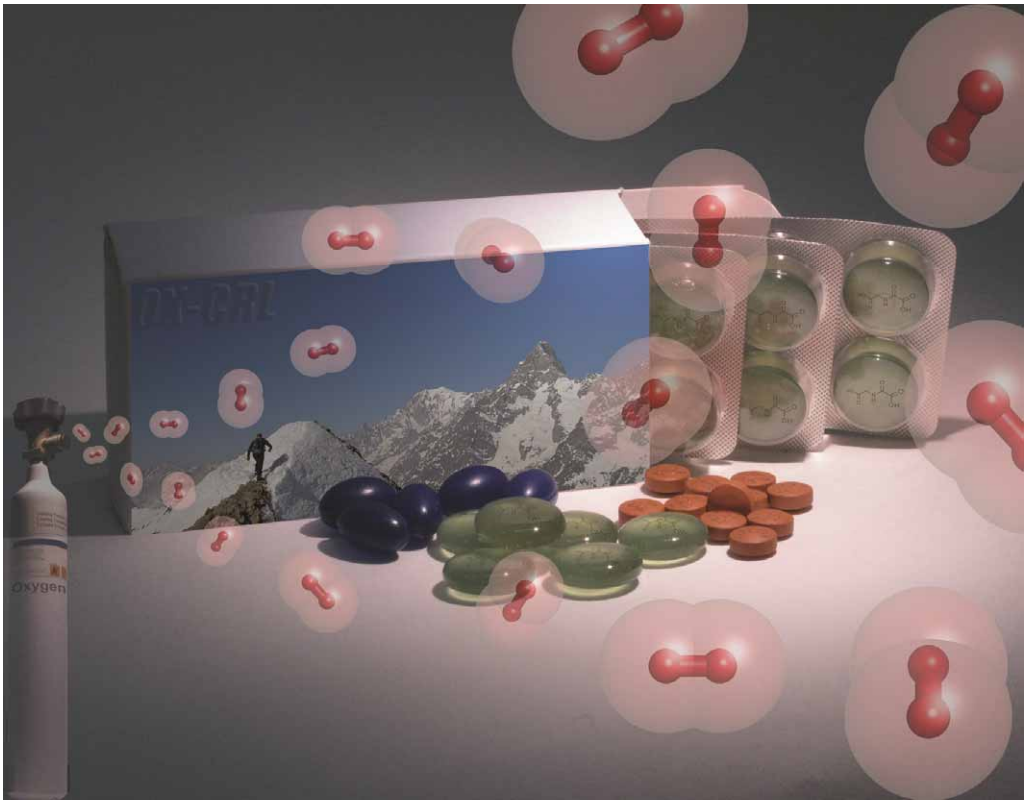

Chemical investigation of light induced DNA bipyrimidine damage and repair†

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In all organisms, genetic information is stored in DNA and RNA. Both of these macromolecules are damaged by many exogenous and endogenous events, with UV irradiation being one of the major sources of damage. The major photolesions formed are the cyclobutane pyrimidine dimers (CPD), pyrimidine–pyrimidone-(6-4)-photoproducts, Dewar valence isomers and, for dehydrated spore DNA, 5-(α-thyminyl)-5,6-dihydrothymine (SP). In order to be able to investigate how nature's repair and tolerance mechanisms protect the integrity of genetic information, oligonucleotides containing sequence and site-specific UV lesions are essential. This *tutorial review* provides an overview of synthetic procedures by which these oligonucleotides can be generated, either through phosphoramidite chemistry or direct irradiation of DNA. Moreover, a brief summary on their usage in analysing repair and tolerance processes as well as their biological effects is provided.

1. Introduction

Nucleic acids (RNA and DNA) are used by organisms for storage of genetic information, which is encoded in the sequences of the four nucleobases adenine, cytosine, guanine and thymine (and uracil in RNA). UV-light is one of the most frequent exogenous influences that harm these nucleic acids, in particular UV-C (<280 nm).[1,2] However, longer wavelength UV light is of higher biological relevance due to the absorption of UV-C by ozone in the upper parts of the atmosphere.[2]

Dimerization through electrocyclic ring formation between two pyrimidines is the most important photoreaction caused by UV-B and UV-C irradiation of DNA in cells (Scheme 1). Cyclobutane pyrimidine dimers (CPD lesions) **1** and pyrimidine–pyrimidone-(6-4)-photoproducts (6-4 lesions) **4**, caused by these reactions, and their secondary products, the Dewar valence isomers (Dewar lesion) **5**, are the most abundant UV lesions and are responsible for much of the destructive effect of UV light. In the formation of CPD lesions the two C5=C6 double bonds react in a [2π + 2π] cycloaddition, whereas a cycloaddition between the C5=C6 double bond of the 5′ nucleoside and the C4 carbonyl group of the 3′ nucleoside, named Paternó–Büchi reaction, leads to (6-4) lesions. The harmful effects of UV-A, which does not directly affect the nucleobases, are due to the excitation of photo sensitizers and further triplet energy transfer resulting in the formation of CPD lesions.

It is also known that UV-C radiation can cause various reactions such as the photohydration of pyrimidines and direct photooxidation of guanine.[1] Adenine, a purine base, also shows photoreactivity, with involvement in cycloaddition reactions under UV-C irradiation. The C5=C6 double bond of a 3′ side adenine can either react with the 5′-side C5=C6 double bond of thymine or with the N7=C8 double bond of

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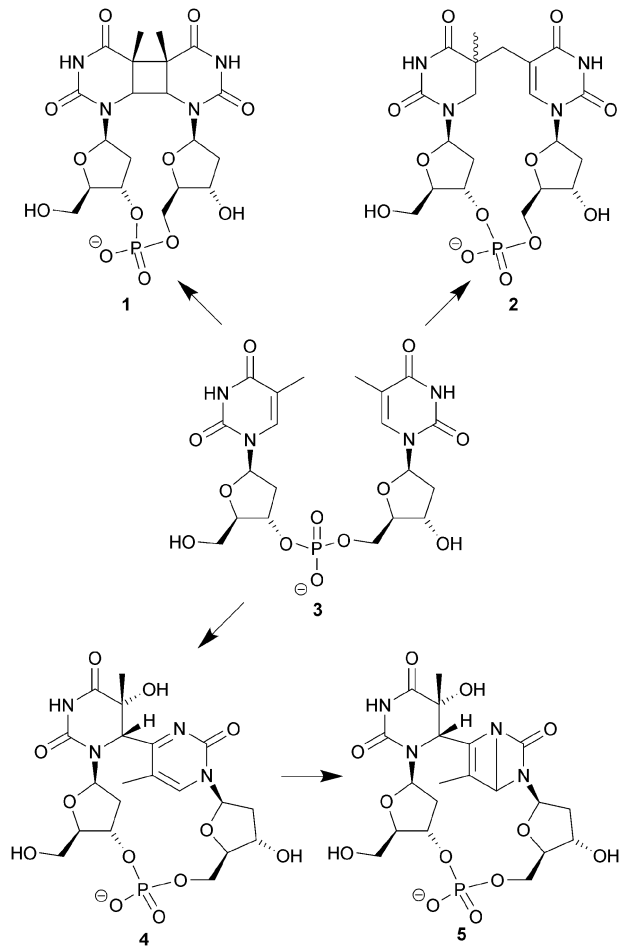

Scheme 1 Depiction of the four main pyrimidine–pyrimidine photolesions. **1**: Cyclobutane pyrimidine dimer, **2**: spore photoproduct, **4**: (6-4) photoproduct, **5**: Dewar valence isomer of **4**.

Thomas Carell

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adenine.[3,4] Secondary reactions result in two different types of AA lesions and, by ring expansion, in the TA lesion.

In addition to the above named lesions a unique dimer is created if bacterial spores are exposed to UV-C, 5-(α-thyminyl)-5,6-dihydrothymine, also known as the spore photoproduct (SP) **2**.[5] This lesion is formed due to complexation of the DNA with small acid soluble proteins (SASPs), a high level of dehydration and the considerable amounts of dipicolinic acid present in spores. This environment significantly changes the photochemistry of DNA and the other photolesions are no longer formed.

Replicative polymerases cannot replicate through UV induced dimers, which therefore stop replication. However, nature has developed several pathways to handle this situation in order to avoid apoptosis. Base excision repair (BER), nucleotide excision repair (NER), photolyase-induced repair and trans lesion synthesis (TLS) are the most important of these.

In BER the *N*-glycosidic bond between a damaged base and its corresponding deoxyribose is hydrolysed *via* a special DNA glycosylase.[6,7] Subsequent processes involving nucleotide insertion and ligation complete the repair. BER is predominantly responsible for the removal of oxidative lesions and deaminated bases, but there are also prokaryotes and lower eukaryotes known in the literature with glycosylases specific for *e.g.* the CPD lesion.[8]

NER is a repair pathway utilised for many different DNA lesions in prokaryotes and eukaryotes.[9,10] Even though the proteins involved vary significantly between species, the general mechanism is similar in all organisms. Initially the lesion is recognised and excised together with several surrounding bases. The resulting gap is then filled up by a polymerase and a ligase seals the nick. There are, depending on the organism, many enzymes involved (from <10 in prokaryotes to at least 30 in eukaryotes such as yeast and humans) and for human NER the picture is not complete. However, it is clear that NER is a very important repair pathway for UV induced lesions, because the lack of a NER enzyme causes *Xeroderma Pigmentosa*, a hereditary disease which results in a high skin cancer rate and increased UV-light sensitivity. The (6-4) and Dewar lesions seem to be good substrates for NER, whereas for the CPD lesions other pathways such as TLS and photolyase-induced repair seem to be more important.

Photolyases are monomers with a molecular weight of about 55–60 kDa and a catalytic $FADH^-$ cofactor which injects electrons for the photoreversion of the dimers to the corresponding undamaged nucleotides.[11] A second cofactor such as 5,10-methylenetetrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyl-5-deazariboflavin (8-HDF) acts as a light-harvesting photoreceptor to improve the quantum yield. There are two types of photolyases, namely those that repair CPD lesions and those that repair (6-4) lesions and their Dewar valence isomers. Surprisingly and still unexplainable, experiments show that T(Dew)T is not repaired by these enzymes, or if so, with an extraordinary low quantum yield.[12,13] Moreover, not all organisms, (*e.g.* higher eukaryotes like humans) are able to perform this type of repair.

Aside from the light driven photolyases, which are related to cryptochromes, there is one light independent photoproduct

repair enzyme, the spore photoproduct lyase, which is a radical *S*-adenosylmethionine (SAM) dependent enzyme.[14] The SP lyase uses an iron–sulfur cluster ([4Fe–4S]) and SAM as cofactors to yield a 5′-deoxyadenosyl radical and finally repair the SP.

The repair and formation of these lesions was and is still a key objective in scientific research, and as synthetic lesions and lesion analogues are powerful tools to investigate these pathways, chemistry hand in hand with biology is necessary to solve these questions of the nature of life.

2. Cyclobutane pyrimidine dimers

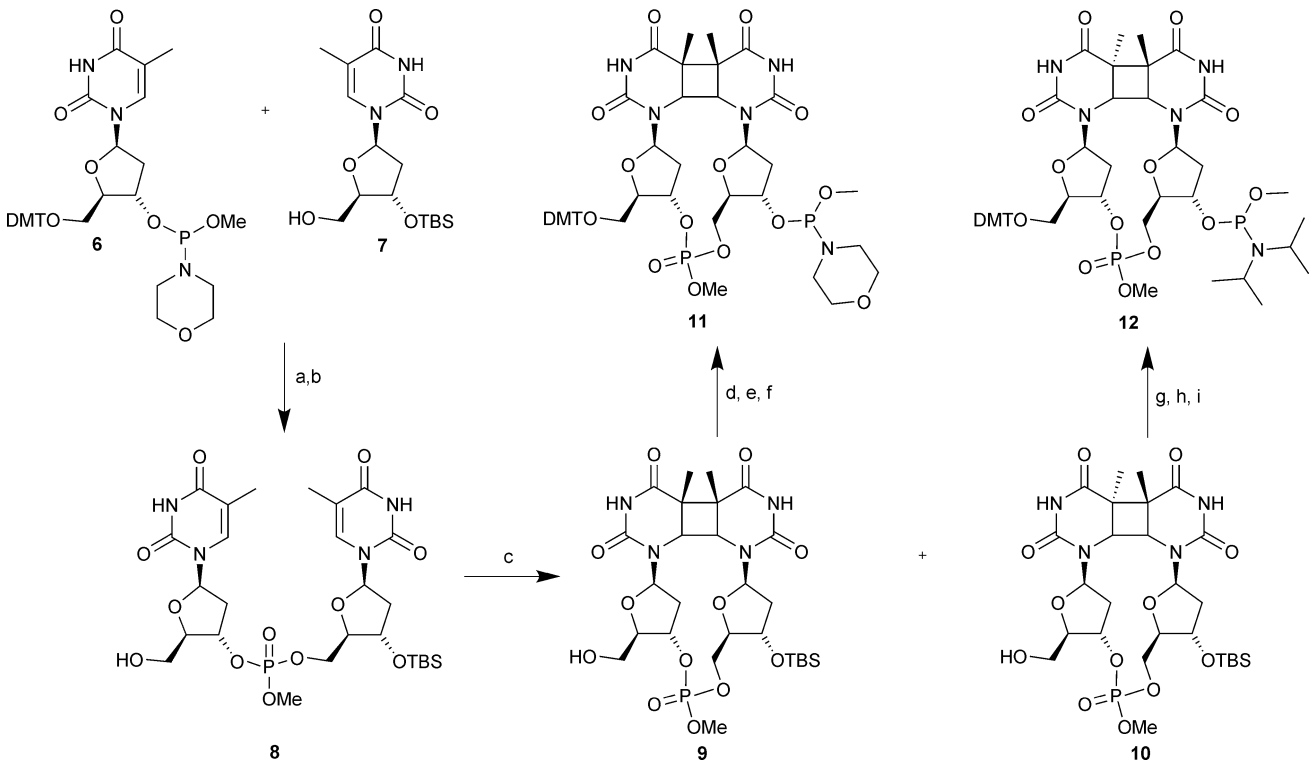
CPDs are the most abundant lesions resulting from UV irradiation and the [2π + 2π] cycloaddition principally gives rise to three different configural isomers of the four-membered ring. However, due to the fixed conformation (*anti* relative to the glycosidic bond) of a DNA double helix, the *cis-syn*-isomer is almost exclusively formed. CPDs appear *in vivo* between all pyrimidine pairs, but not in equal ratios. The formation trends are 5′-T(CPD)T-3′ > 5′-T(CPD)C-3′ > 5′-C(CPD)T-3′ > 5′-C(CPD)C-3′, where the yield of T(CPD)T is about three times higher than T(CPD)C.[15,16] It is worth mentioning that the NH_2 substituents of CPDs are not stable and deaminate spontaneously to form uracil-containing secondary photoproducts in an hour timescale.[17,18]

The first synthesis of T(CPD)T (Scheme 2), including incorporation into DNA as a phosphoramidite, was reported by Taylor *et al*. in 1987.[19] The dinucleotide **8** was synthesized by coupling of the thymidine phosphoramidite **6** and the 3′-silyl protected thymidine **7** with tetrazole as activator, followed by oxidation with iodine to afford the desired phosphorus(v) species. After deprotection of the 5′-OH, irradiation with Pyrex-filtered UV light in the presence of acetophenone as triplet sensitizer led to the partially protected CPD **9** as well as its configural isomers. Both *cis-syn* CPD diastereomers, epimeric at phosphorus, were separated by HPLC. Protection of **9** with DMTCl, followed by removal of the *tert*-butyldimethylsilyl group and reaction with chloro(methoxy)(morpholinyl)phosphine led to the phosphoramidite building block **11**. The oligonucleotide, synthesized on controlled pore glass, was deprotected and cleaved from the solid support with a two step protocol, necessary due to the removal of the methoxy group at the phosphorus.

The *trans-syn* isomer was incorporated in DNA with a similar strategy.[20] The conversion of **10** was done with a modified procedure including reversal of the tritylation and desilylation steps and replacement of the morpholinyl-phosphoramidite by the more reactive diisopropylaminophosphoramidite to yield **12**.

A more elegant synthesis based on cyanoethyl and levulinyl protecting groups was developed by Ohtsuka and coworkers.[21] The cyanoethyl protecting group on the phosphate allows the use of a one step protocol for deprotection and cleavage.

Since the levulinyl group can be removed in the presence of a cyanoethyl protected phosphate, it was also possible to synthesize T(CPD)T dithionate building blocks. These dithionate substrates, as well as CPD lesions, with methyl protection at the phosphorus, were used to clarify the binding mode of T4 endonuclease V, a CPD specific glycosylase.

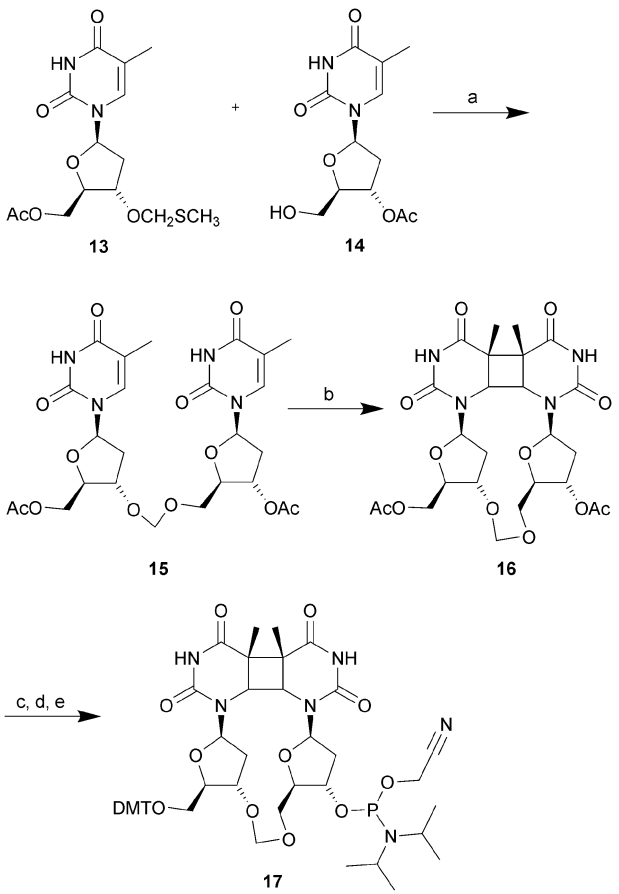


Scheme 2 Synthesis of *cis-syn* and *trans-syn* CPD lesion **11** and **12**: (a) 1*H*-tetrazole, MeCN then I_2 in H_2O, pyridine; (b) *e.g.* 80% HOAc; (c) H_2O, MeCN, acetophenone, hυ (450 W Hg-lamp); (d) DMTCl, pyridine; (e) TBAF, THF, HOAc; (f) morpholine, CH_3OPCl_2, DIEA; (g) HCl, acetic acid; (h) DMTCl, pyridine; (i) $CH_3OPN(i\text{-}Pr)Cl$, DIEA.

These studies showed that the enzyme interacts strongly with its substrate in the minor groove of DNA, in agreement with a later crystal structure.[8,22]

The usage of a phosphodiester bridged photolesion requires protection as illustrated, which results in additional reaction steps and leads to the formation of an additional stereocenter at the phosphorus resulting in the necessity of HPLC to separate the diastereomers.

To overcome these problems Carell *et al.* developed the synthesis of a bio-isosteric formacetal linked T(CPD)T.[23] For this purpose the 5′-*O*-acetyl-3′-*O*(methylthiomethyl)-protected thymidine **13** was coupled with **14** to give the formacetal bridged dinucleotide **15** (see Scheme 3). Irradiation of **15** in a Pyrex device resulted in a mixture of the *cis-syn* **16** and the *trans-syn* isomers. In this reaction mixture only the formacetal linked *cis-syn* T(CPD)T is insoluble in acetone, which allows the purification of the desired lesion in multigram scale simply by washing off the precipitate. After deprotection and two-step conversion to the DMT protected phosphoramidite **17**, solid phase synthesis of oligonucleotides under standard conditions is possible. The U(CPD)U lesion was synthesized using the same route. For these uridine derivatives normal deprotection of oligonucleotides with conc. NH_4OH resulted in opening of the C(4)–N(3) bond, but the use of saturated ammonia in anhydrous methanol allowed the preparation of U(CPD)U-containing oligonucleotides in excellent yields.[23]



Scheme 3 Synthesis of T(CPD)T with formacetal backbone **17**, (a) NIS, TfOH, THF; (b) hυ (150 W Hg-lamp), acetone; (c) NH_4OH, rt, 12 h; (d) DMTCl, pyridine, DIEA; (e) $(NCCH_2CH_2O)PN(i\text{-}Pr)_2(Cl)$, DIEA, THF.

The formacetal bridged T(CPD)T was used, for example, in the first reported crystal structure of a photolyase (*Anacystis nidulans* CPD photolyase) in complex with UV damaged DNA.[24] The oligonucleotide is bent by 50° and the lesion is flipped into the active side of the enzyme, where it was split into two thymidines by the synchrotron radiation at 100 K. This structure mimics a substrate during light-driven DNA repair in which back flipping of the thymines has not yet occurred, which demonstrates that substitution of the phosphodiester group by an uncharged, hydrophobic formacetal does not affect binding properties significantly.

As mentioned previously, higher eukaryotes (*e.g.* humans) are unable to perform this type of repair and use different pathways such as transcription-coupled repair (TCR), which is a very efficient NER subpathway, to remove this type of lesion.[25] Cramer and co-workers used formacetal bridged T(CPD)T for structure-based investigation of the mechanism of the first step in eukaryotic TCR, CPD-induced stalling of RNA polymerase (Pol) II.[26] The lesion slowly passes the bridge helix (Fig. 1) and enters the active site where the 5′-thymine of the CPD then directs uridine misincorporation into mRNA, which blocks translocation. The inaccessibility of the lesion in the stalled complex and the unchanged conformation of the polymerase indicate nonallosteric recruitment of other TCR proteins and excision of the damaged oligonucleotide in the presence of Pol II.

Aside from these examples, oligonucleotides containing synthetic T(CPD)T lesions are extensively used in studies to clarify UV lesion repair, particularly as the T(CPD)T phosphoramidite is commercially available. However for special requirements, particular UV lesions such as a T(CPD)T with an open backbone need to be synthesized.[27–29] For this purpose, CPD phosphoramidites, such as **18**, where the internal phosphate is replaced by a base cleavable group such as a

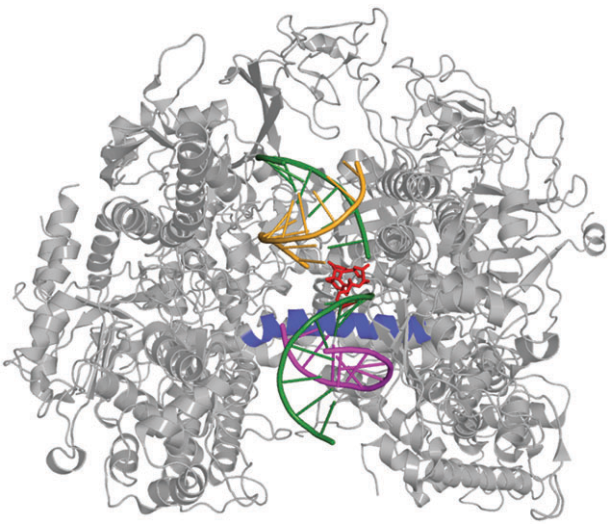
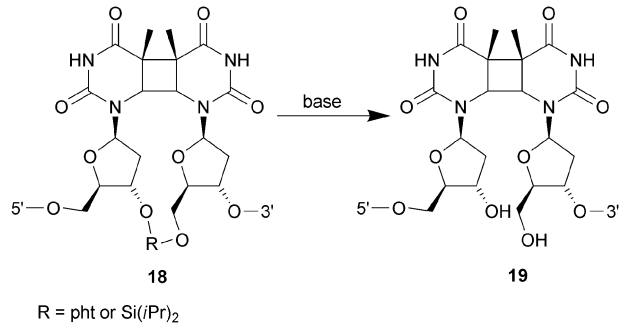


Fig. 1 Pol II elongation complex structures with T(CPD)T in the template. Protein is in gray, the bridge helix in blue, and the lesion is shown in red. A large portion of the second largest Pol II subunit was omitted for clarity.



Scheme 4 Formation of T(CPD)T with open backbone during cleavage of oligonucleotides from solid support.

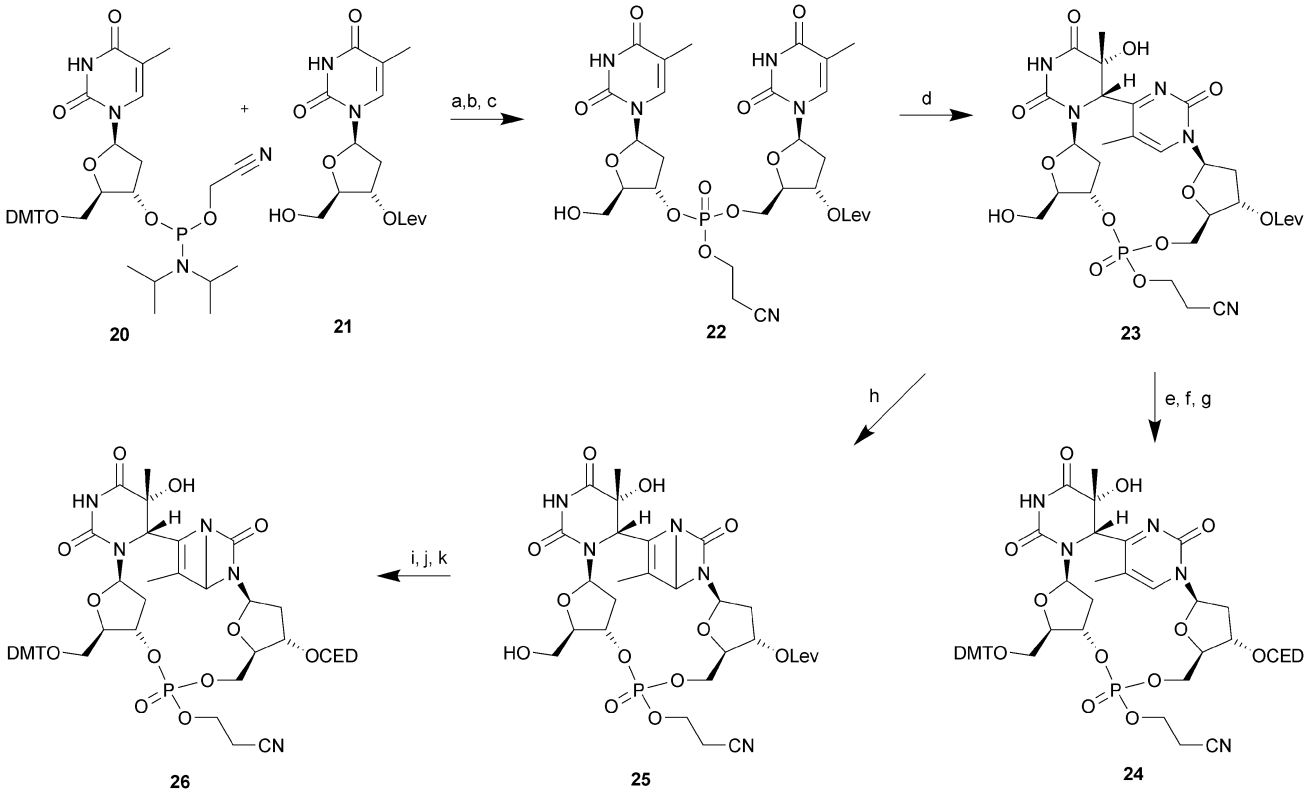
phthalic acid ester or a diisopropylsilyl group can be used (see Scheme 4).[27,29] This group is removed during cleavage from the solid support and removal of the other protecting groups to give compounds as **19** in the duplex. In assays with photolyases and for studying electron transfer processes the CPD **19** is converted to two thymidines, resulting in an easily detectable strand break.[28]

3. Pyrimidine–pyrimidone-(6-4)-photoproducts and Dewar valence isomers

As mentioned previously, (6-4) lesions are also formed by a photochemical formal [2π + 2π] cycloaddition. Here, the C5═C6 double bond of the 5′ nucleoside reacts, possibly *via* a triplet state, with the C4 carbonyl group of the 3′ nucleoside. In the case of two thymidines, this occurs in a Paternó–Büchi reaction to give an oxetane. This oxetane, which is not stable above −80 °C, reacts further by ring opening to give the (6-4) photoproduct. In the case of a 3′ cytidine the reaction occurs from an imine tautomer with formation of a azetidine intermediate. (6-4) Lesions occur more efficiently on TC rather than TT sites. Reactions with a 5′ cytidine are also possible, however they are the least frequent.[30]

On exposure to additional UV-A irradiation, (6-4) lesions undergo a formal 4π electrocyclic rearrangement to give the corresponding Dewar valence isomers, which are similar in many aspects to (6-4) lesions. Dewar lesions are, in fact, at least as important as their parent lesions as they are more abundant than (6-4) lesions in DNA of cells irradiated with simulated or natural sunlight.[16,31]

The first reported (6-4) lesion in oligonucleotides was made using a synthetic approach based on a levulinyl cyanoethyl protecting group strategy (Scheme 5).[32] Coupling of the 5′-*O*-DMT-cyanoethylphosphoramidite **20** with the 3′-*O*-Lev-protected cytidine **21**, oxidation of the bridging phosphite with iodine and deprotection with acetic acid afforded the T–T-dinucleotide **22**. Irradiation with UV-C light from germicidal mercury lamps under anaerobic conditions led to a mixture of photoproducts from which the (6-4) dinucleotide **23** was isolated in yields of about 15% in the best cases. The 5′-hydroxyl group was converted into the DMT protected species and the 3′-OH of this compound was then deprotected with hydrazine and converted to the phosphoramidite **24**. Due to high steric hindrance,



Scheme 5 Synthesis of T(6-4)T and T(Dew)T building blocks **24** *and* **26**. (a) 1*H*-tetrazole; (b) I_2, H_2O, pyridine; (c) 80% HOAc; (d) UV (254 nm), 20% MeCN; (e) DMTCl, pyridine; (f) $NH_2NH_2{\cdot}H_2O$, pyridine, 5 min; (g) $(NCCH_2CH_2O)PN(i\text{-}Pr)_2Cl$, DIEA; (h) UV (>280 nm); (i) DMTCl, pyridine; (j) $NH_2NH_2{\cdot}H_2O$, pyridine, 5 min; (k) $(NCCH_2CH_2O)PN(i\text{-}Pr)_2Cl$, DIEA.

the C(5)–OH needs no protection for solid phase DNA synthesis.

The strategy of levulinyl cyanoethyl protection was also used by Iwai *et al*. to synthesize the T(6-4)C and the T(Dew)T phosphoramidites.[33,34] In the case of the T(6-4)C building block the C(5)–NH_2 was protected with a DMT-group before coupling of the 5′-thymidine and the 3′-cytidine, followed by deprotection then irradiation. HPLC separation of the T(6-4)C and three additional reaction steps yielded the desired phosphoramidite. It was found that in standard solid phase synthesis with this compound, acetylation of the exocyclic amino group occurs during the capping step, which makes it necessary to omit this step after coupling of the (6-4) unit. For the synthesis of the Dewar phosphoramidite the 5′-unprotected T(6-4)T **23** was prepared as mentioned previously.[32] Irradiation with Pyrex filtered UV light, followed by HPLC purification yielded the partially protected T(Dew)T **25** which was converted to the phosphoramidite **26** as previously.

In addition to the natural lesions, modified (6-4) and Dewar lesions have been used. For example, Fourrey and coworkers used T (6–4)s^{5}T, produced by irradiation of 5′-*O*-thymidylyl-4-thiothymidine, for mechanistic studies, which indicated that repair by (6-4) photolyases proceeds *via* a oxetane such as azetidine.[35] Furthermore, a synthetic route to the phosphoramidite of this thio analogue is available.[36]

Although the toolset for investigation of (6-4) and Dewar repair with synthesized lesions and oligonucleotides is available, this approach is used in very few cases, as synthesis of the phosphoramidite building blocks is lengthy, difficult and the efficiency unsatisfactory. Most biological studies are done with lesion-containing DNA produced by direct irradiation of oligonucleotides. The unique absorption of (6-4) lesions at about 320 nm enables detection and isolation of the desired oligonucleotides even from complicated irradiation mixtures and conversion to the Dewar isomers is typically clean and complete. Early experiments were only able to isolate short oligonucleotides containing T(6-4)T such as T(Dew)T.[37,38] However, careful choice of the nucleotide sequence and optimized anaerobic irradiation conditions enable the irradiation of 18mers without difficulty in high purity and satisfactory yields.[39] The limitation in length can furthermore be overcome by ligating short irradiated oligonucleotides into larger sequences.[40]

With these irradiated oligonucleotides, the crystal structures of *Drosophila melanogaster* (6-4) photolyase in complex with oligonucleotides containing its natural substrates T(6-4)T and T(6-4)C were obtained (Fig. 2).[39,41] These structures provide support for a mechanism without rearrangement of the (6-4) lesions to strained four-membered ring intermediates and argues for a direct electron injection into the lesion as the initial step of repair performed by (6-4) DNA photolyases.[42]

The use of irradiated oligonucleotides was also successfully used for the production of unnatural (6-4) lesions. Carell and coworkers showed that the use of N(4)-methylcytosine (C*) in thymidylyl(3′–5′)cytidine sequences results in T(6-4)C* lesions with amazingly low repair rates, with a surprising scientific outcome.[43] During repair of T(Dew)C*, bearing the exocyclic –NHMe group, the low turnover of the corresponding (6-4) lesion leads to accumulation of it as an intermediate in the repair reaction. Further experiments with oxidized FAD indicate that this rearrangement requires electron injection. Therefore it was shown that (6-4) photolyases repair Dewar valence isomers by electron driven isomerisation to (6-4) lesions.[43]

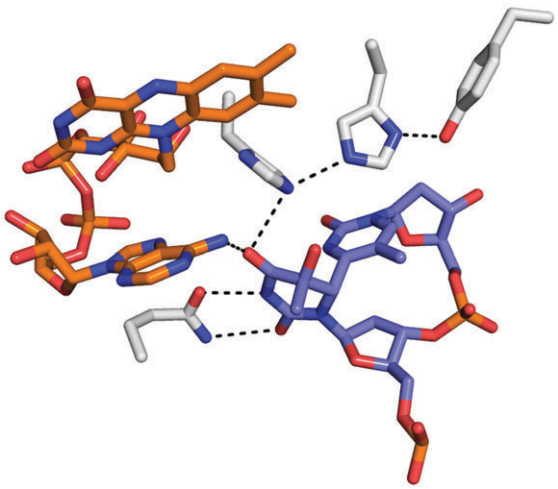


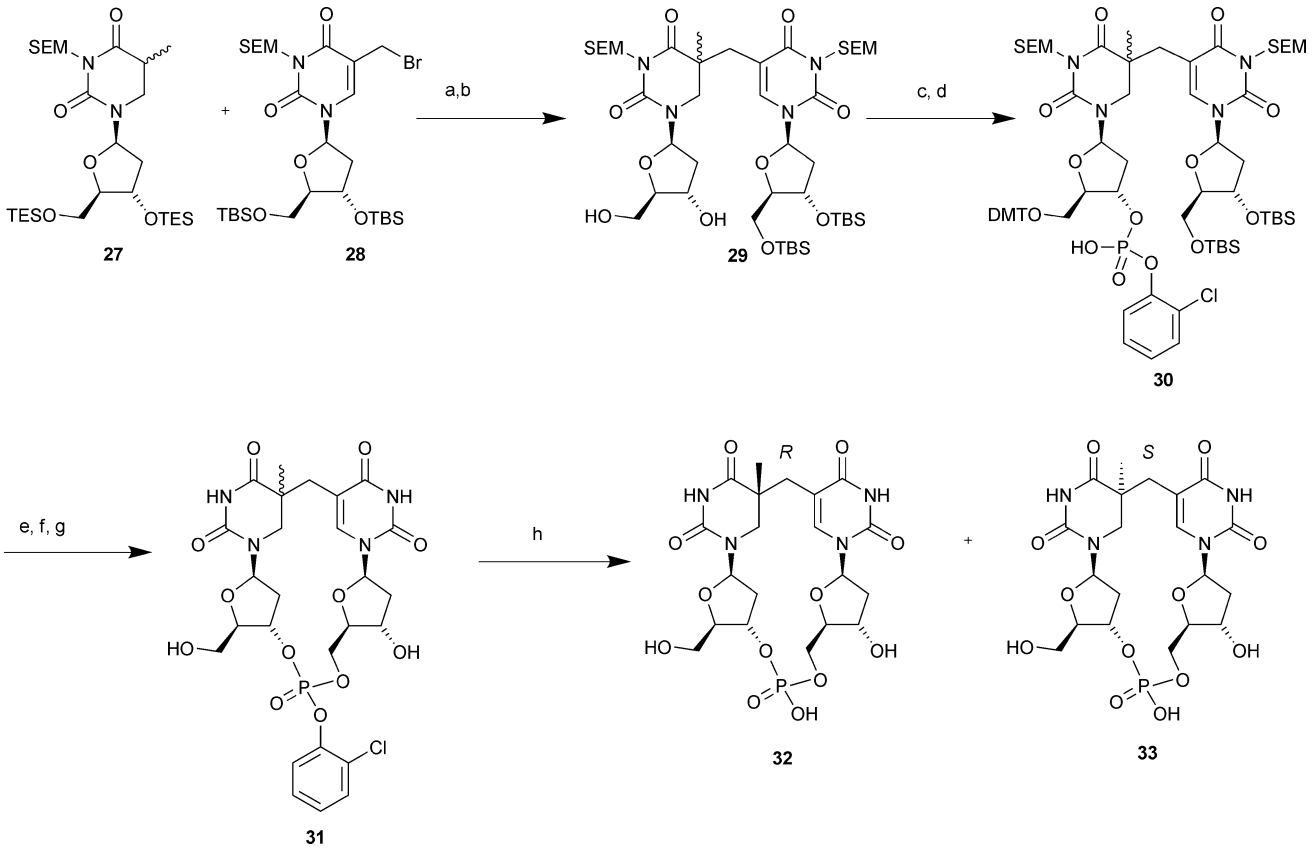
Fig. 2 The active site structure of the (6-4) photolyase. The FAD is shown in orange, with selected active site residues shown in grey.

4. The spore photoproduct

The previously mentioned spore photoproduct (SP), discovered in 1965 by Donnellan and Setlow, is in many aspects different to the CPD, (6-4) and Dewar lesions.[5] However, the SP is not formed *via* an electrocyclic reaction and is uniquely found in spores, where it is the predominant lesion and only exists as T(SP)T. Formation, synthesis and repair of the lesion have been recently reviewed and therefore only key issues will be presented here.[14]

Begley and coworkers developed the first total synthesis of 5-(α-thyminyl)-5,6-dihydrothymine (see Scheme 6).[44] The key step of this synthesis is the enol coupling of the allylbromide **27** and the 5,6-dihydrothymidine **28**. In this step **27** is lithiated with LDA, then added to **28**, followed by removal of the TES groups with HF in acetonitrile to afford **29** as a diastereotopic mixture. After treatment with DMTCl to protect the 5′-OH group, 2-chlorophenyl-dichlorophosphate together with 1,2,4-triazole was used to yield the protected phosphate **30**. TBAF deprotection and 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole mediated ring closing in pyridine furnished the phosphotriester linked dinucleotide. Treatment with $SnCl_4$ simultaneously cleaved the SEM and DMT groups to give **31**, which was finally deprotected with NH_4OH to afford the two diastereomers **32** and **33** after HPLC separation.

Solid phase synthesis of DNA containing a SP analogue with an open backbone has been recently published.[45] The key step is again an enol coupling between a dihydrothymidine and an allylbromide, whereas the choice of protecting groups is different in order to replace the internal phosphate by two TBDPS groups. A three step protocol was used to obtain oligonucleotides containing the SP analogue after solid phase synthesis with the corresponding phosphoramidite. Removal



Scheme 6 (a) LDA, THF, −78 °C–0 °C; (b) 4% HF in MeCN; (c) DMTCl, TEA, pyridine; (d) 2-chlorophenyl-dichlorophosphate, 1,2,4-triazole, THF; (e) Bu_4NF, THF; (f) MSNT, pyridine; (g) $SnCl_4$, CCl_4; (h) NH_4OH, rt.

of the *N*(3)-SEM groups was achieved by treatment of the DNA strand with 1 M $SnCl_4$ in THF on the solid support, followed by cleavage from the support and removal of the TBDPS groups with TBAF.

For enzymatic assays the SP-containing DNA and the SP dinucleotide are normally produced *via* irradiation of dried oligonucleotides together with dipicolinic acid (DPA).[5,14,16,46–49] However, in order to obtain defined nucleotide sequences, necessary for many applications such as crystallization, this method is limited to short oligonucleotides and the yields are very unsatisfactory.

Nicholson and coworkers used such oligonucleotides to investigate the binding mode of spore photoproduct lyase.[50] The SP containing DNA shows enhanced digestion by DNase I if SP lyase is added. These data suggest that SP lyase causes a significant bending or distortion of the DNA helix such as base flipping in the vicinity of the lesion.

The mode of repair of the spore photoproduct by SP lyase is still unclear and even the question of which diastereoisomer is finally accepted as a substrate has not been fully clarified.[51–53] Indeed, a mechanism has been proposed and is supported by experiments such as mutation studies on SP lyase, but structural information such as crystal structures is still lacking.[54,55]

5. Conclusions

Investigation of nature's mechanisms of photolesion recognition, tolerance and repair is limited and directly linked to the availability of lesion-containing DNA. Currently this availability varies considerably and is highly dependent on the type of lesion. For instance, CPD building blocks for solid phase synthesis of DNA are state of the art and commercially accessible, thus making this lesion the best understood example of UV induced DNA damage. In contrast, generation of (6-4) and Dewar lesion bearing oligonucleotides is still a challenging task. The synthesis of building blocks suitable for solid phase synthesis is long with unsatisfying yields, whereas direct irradiation techniques are limited to special sequences and relatively short oligonucleotides. Moreover, an efficient route to SP containing DNA with defined sequence and length is still missing. Direct irradiation protocols suffer from very low yields, complicated purification and limitation to very short oligonucleotides. No synthesis of a phosphoramidite building block of the natural SP is available yet. Only a SP analogue with an open backbone can be obtained, the synthesis of which is time-consuming, not stereoselective and therefore requiring the separation of diastereomers.

Thus, while the atomic details of the recognition and light-driven repair of CPD and 6-4 lesions are known, knowledge on the mechanism and function of the light-independent SP lyase are only rudimentary. SP repair together with the more general tolerance mechanisms TLS and NER seem to be the main subjects of further investigation into UV lesion repair.

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