Repair of UV Light Induced DNA Lesions: A Comparative Study with Model Compounds

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DNA photolyases are enzymes that catalyze the light-dependend repair of *cis-syn*-cyclobutane-thymine dimer UV lesions in a variety of organisms. The basis of the repairreaction is an electron transfer from a reduced and deprotonated flavin cofactor to the dimer unit, which splits spontaneously as its radical anion. A second cofactor, which is either an 8-hydroxy-5-deazaflavin or a methenyltetrahydrofolate is required as a photo antenna and ensures efficient light absorption. With the help of model compounds that are able to mimic all crucial steps of the repair reaction, detailed mechanistic insights into the repair reaction could be obtained. It became clear, that the enzyme requires the reduced flavin in its deprotonated form and that the repair reaction proceeds most efficiently in polar media, which is in agreement with the observed highly polar flavin binding pocket. Investigations with flavin- and deazaflavin-containing model compounds confirmed that the deazaflavin functions solely as a photo antenna and allowed to study the dependencies of the antenna function on the protonation state of the 8-hydroxy-5-deazaflavin. The ability to mimic the repair reaction with small model compounds allowed finally the development of flavin cofactor functionalized oligopeptides. Cofactor peptides with the sequence of the DNA-binding domain of the transcription factor MyoD were shown to be able to repair UV light lesions of DNA within a DNA single strand.

1. Introduction

The sequence of the four canonical bases thymine, adenine, guanine and cytosine, in the form of a DNA double strand, stored inside cells, represents the genetic information in higher organisms.[1] The base sequence determines all life processes including cell division (replication).[2] Degradation reactions and the misincorporation of bases during cell division are inevitable and occur frequently in a large and actively replicated genome.[3] These damaging processes cause the appearance of mispaired bases[4] and of truncated or dimeric nucleobases within the

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MICROREVIEWS: This feature introduces the readers to the authors' research through a concise overview of the selected topic. Reference to important work from others in the field is included.

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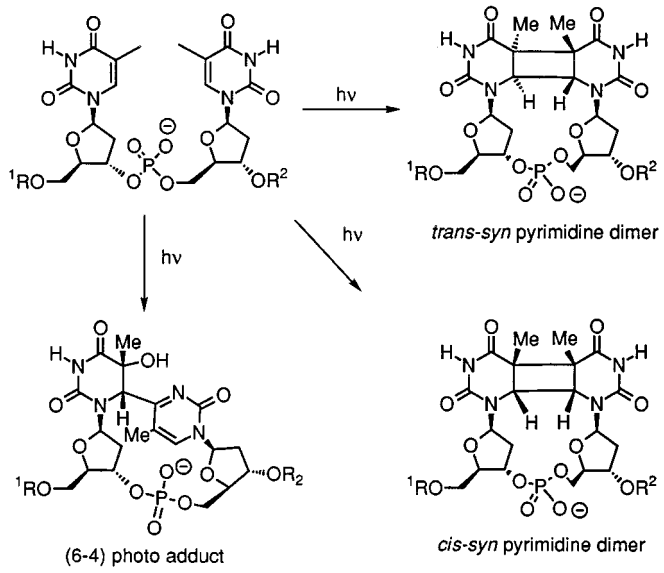
DNA double strand.[5] In addition, complete nucleobase losses occur, which generates apurinic and apyrimidinic sites. Each cell is consequently experiencing a continuous loss of genetic information, which disturbs the function of critical cellular processes.[6] All these DNA damages are often triggered by either endogenous factors like reactive OH radicals[7], generated in the course of metabolic processes, or by exogenous events like γ or UV irradiation.[8] Some of the structures of the resulting base degradation products are known, and in a few cases the presence of particular lesions can be associated with specific cellular misfunctions. Some DNA lesions have been directly linked to the process of tumor genesis.[9][10] The result of UV irradiation, which is the focus of this review article, is the formation of cyclobutane-pyrimidine dimers (Figure 1) through a [2π + 2π] cycloaddition reaction between two pyrimidine bases located above each other in double-stranded DNA.[11][12][13] The main photo adducts are the *cis-syn*-configured compounds. The corresponding *trans-syn* lesions are formed to a much smaller extent, predominantly upon irradiation of single-stranded DNA. These defects represent a particularly serious threat to organisms that possess a single-stranded genome during their life cycle.[14] A third deleterious UV light induced lesion is the [6,4] photo adduct, which is formed in a Parterno-Büchi reaction between two adjacent pyrimidine bases. All three types of UV lesions are thought to be responsible for the appearance of basal and squamous skin cell carcinomas and maybe for the development of malignant melanoma.[15][16][17][18]

Figure 1. Structures of the most abundant DNA lesions formed upon UV irradiation of double- and single-stranded DNA[9][10]



All organisms developed efficient DNA repair functions in order to counteract the lethal effect of these lesion formation reactions.[19] Specialized repair proteins scan the genome continuously for the presence of DNA lesions. Once a lesion recognition protein like XPA[20] − in humans − encounters a mispaired base, an apurinic or apyrimidininic site, or structurally altered bases, it triggers an efficient DNA repair, which ultimately leads to the restoration of the genetic information. The above-mentioned UV lesions are in many species directly excised from the genome in repair processes called nucleotide[21] or base excision repair.[19] Other species − most probably not humans − are able to split the most abundant *cis-syn* dimer lesions directly back into the monomers with the help of the DNA repair enzyme DNA photolyase (Figure 2).[22][23] This enzyme catalyzes the thermally Woodward-Hoffmann forbidden [2π + 2π] cycloreversion reaction. Since DNA photolyases are found in a variety of archaebacteria,[24] they can be considered to be ancient repair proteins, which may have already helped the development of the earliest organisms on the primordial earth. Such early organisms were exposed to high UV irradiation levels, due to a then missing ozone layer. It is a fact that the ozone layer, which now shields the earth from UV light, is currently experiencing continuous depletion,[25][26] thus yielding increasing UV levels and skin cancer incidents, including malignant melanoma. This current threat has recently fueled the interest to gain understanding of the DNA photolyase mechanism on a molecular level.[27] The ultimate goal could be the development of

Figure 2. Ribbon representation of the DNA photolyase from *A. nidulans* (based on X-ray data)[30]; the two cofactors FAD and 8-HDF are shown as stick models (FAD: red, 8-HDF: yellow)

artificial DNA photolyases, that are able to simulate the efficient recognition and repair of DNA UV lesions in vivo. Such compounds might also bring relief to patients suffering from *xeroderma pigmentosum* or *trichothiodystrophy.* Both diseases are inherited human disorders associated with a reduced ability to excise DNA lesions from the genome.[15][19]

Since the enzymatic investigations, which led to the currently accepted DNA photolyase repair mechanism, are the subject of several excellent recent reviews,[22][23][28] this article's focus is to summarize the progress in our effort to learn about the photolyase mechanism with the help of model compounds. Their synthesis, the performed investigations and the derived mechanistic insights will be discussed in context with the two recently determined X-ray crystal structures of DNA photolyases from *E. coli*[29] and *A. nidulans.*[30] A ribbon representation of the type-II DNA photolyase from *A. nidulans*, showing the essential flavin and deazaflavin cofactors and the overall architecture of the repair enzyme is given in Figure 2.

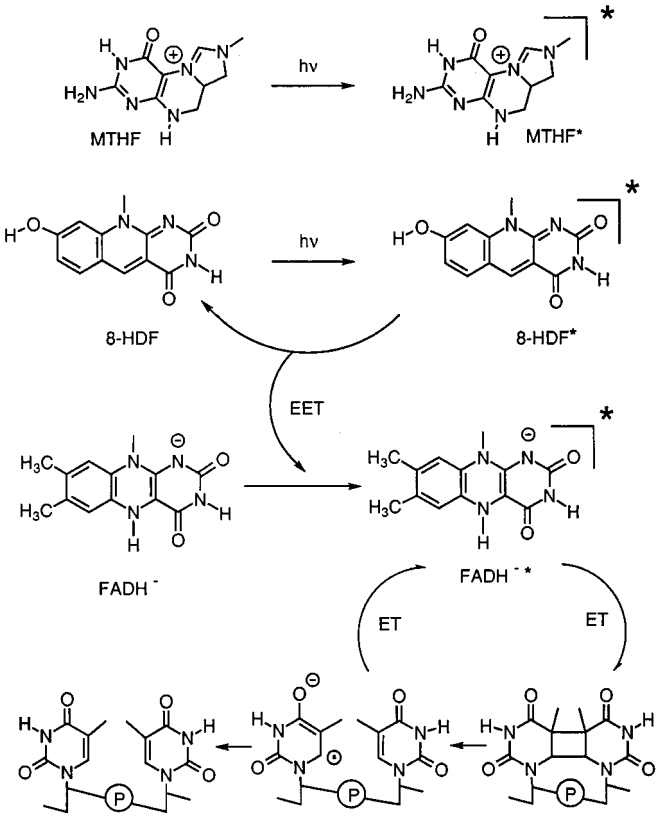
1.1 Mechanism of the DNA Photolyase Catalyzed Genome Repair

DNA photolyases are monomeric flavin-dependent repair enzymes with a molecular weight between 55 and 65 kDa. 10−20 enzyme molecules are believed to scan the genome for UV lesions in every cell nucleus. Each one binds tightly to an encountered *cis-syn*-cyclobutane-pyrimidine dimer with a $K_d = 10^{-8}$ M (thymine dimer). The subsequent splitting reaction requires sunlight with wavelengths of λ = 300−500 nm. Photons possessing these wavelengths are initially absorbed by either a methenyltetrahydrofolate (MTHF) cofactor in type-I photolyases or an 8-hydroxy-5-deazaflavin (8-HDF) cofactor in type-II photolyases (Figure 3). This so-called second cofactor is thought to subsequently transfer the light energy to a crucial FAD cofactor,[31][32][33] present in all known photolyases.[22]

Elegant enzymatic and model compound investigations, performed predominantly by the groups of A. Sancar, M. S. Jorns, S. D. Rose, C. Walsh, A. Yasui, and A. P. M. Eker showed recently that $FADH^-$ is required for the repair.[34][35] By light induction, the $FADH^-$ transfers one electron to the cyclobutane-pyrimidine dimer unit which is believed to be bound in close vicinity to the $FADH^-$. The formed dimer radical anion splits spontaneously into the monomers and the surplus electron is ultimately transferred back to the flavin radical intermediate (Figure 3).[23][36]

Although the general light-driven splitting scenario, schematically depicted in Figure 3, is well understood, several crucial aspects of the repair process remain mysterious. (A) It is currently unclear how repair enzymes − including DNA photolyases − recognize single DNA lesions with high precision in a structurally heterogeneous megabase prokaryotic or even chromatin-containing eukaryotic genome.[37][38][39] (B) The process which leads to the initial reduction of FAD to $FADH^-$ remains unknown. Based on site-directed mutagenesis studies[40] and on ESR investigations[41], it was suggested that the formation of $FADH^-$ results from a temporary photo reduction and requires an

Figure 3. Mechanism of the UV light lesion repair of DNA photolyases[11][23][27] (ET = electron transfer, EET = excitation energy transfer)



electron transfer from a distant tryptophane (Trp^{306} in *E. coli,* distance to the FAD: 13 A!) to the light-excited FAD radical quartet state. (C) It is currently unclear how the enzymes mediate the energy and electron transfer processes in order to achieve repair with almost maximal efficiency (quantum yield ϕ = 0.7−0.9). It is possible that the enzyme has to stabilize potentially short-lived intermediates, in order to increase the lifetime of the "charge separated" state as a prerequisite for the efficient dimer cleavage. (D) In the absence of any knowledge of how photolyases recognize their substrate, the differerent cleavage rates observed for uracil and thymine dimers possessing different configurations and constitutions remain mysterious.[14][42] All these questions can be investigated with model compounds that are able to simulate the repair process.

2. Model Investigations with Flavin Photosensitizers

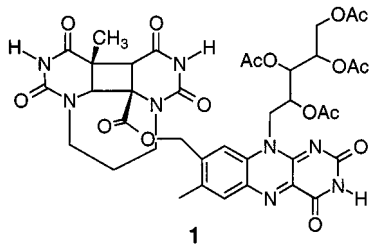
2.1 Previous Experiments with Flavin and Flavin Dimer Solutions

Much of our knowledge about the cleavage of pyrimidine photo dimers has come in the past from model studies. Such model investigations are particularly useful when the uncatalyzed reaction is poorly understood as for example cytochrome P450 catalyzed oxidations,[43] vitamin B_{12} performed methylations,[44] electron transfer reactions over long distances as seen in the photosynthetic reaction center,[45][46][47] or the reactions catalyzed by thiaminediphosphate (vitamin B_1).[48][49] Model studies aimed at the

investigation of the flavin-initiated pyrimidine dimer splitting revealed early that the dimer can be split photo-induced as its radical cation, in the presence of oxidizing photosensitizers such as anthraquinone sulfate[50], 9,10-dicyanoanthracene, *p*-chloranil[51][52], 1,4-dicyanobenzene or as its radical anion in the presence of electron-donating photosensitizers such as 2-methylindole[53] or 1,4-dimethoxybenzene derivatives. These observations initially raised the question which of the two mechanisms photolyases would employ for their efficient genome repair.

One of the first experiments with flavin photosensitizers were performed by A. A. Lamola[54], who observed that oxidized flavins are unable to cleave photo dimers at neutral pH. A systematic investigation by C. Walsh and co-workers with a variety of flavins and 5-deazaflavins revealed that an inefficient light-induced cleavage of pyrimidine dimers with flavins as photo sensitizers is possible but requires very high pH values (pH > 10).[55] Under these conditions, significant deprotonation of the dimer unit occurs (pK_a = 10.7),[56] which led to the proposal that electron transfer from the deprotonated dimer to the oxidized, light-excited flavin could be the initial reaction step.

Irradiation of the covalently linked flavin dimer model compound **1**, prepared by S. D. Rose and co-workers showed, that flavins are able to photo-split dimers under very acidic conditions as well.[57] Addition of perchloric acid[58] to the model compound solution is strictly required for the dimer cleavage. Since perchloric acid will readily protonate the oxidized flavin chromophore (pK_a = 0)[59], the dimer cleavage was explained by the potential ability of the light-excited, protonated flavin (FlH^+) to abstract an electron from the dimer, which presumably splits as the radical cation ($Dimer^{\bullet+}$).



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Similar observations were also reported by C. Pac and co-workers, who showed that the irradiation of solutions containing 1,3-dimethylthymine and tetraacetylflavin in the presence of perchloric acid yields rapid photo splitting.[60] The presence of molecular oxygen was observed to accelerate the photo-induced cleavage because it forms an activated complex with the dimer substrate.[61] The same authors reported that perchloric acid can be replaced with magnesium perchlorate.[62] In this case the presence of a (flavin)magnesium complex is assumed to be the photo-active cleavage agent. The authors argue that the electron-deficient (flavin)magnesium complex might, in a light-induced process, abstract an electron from the dimer unit, which cleaves spontaneously as the dimer radical cation ($Dimer^{\bullet+}$).

Stimulated by the observation that the enzymatic dimer cleavage might require the reduced FAD cofactor, further model studies performed by M. S. Jorns[63] were aimed to support this hypothesis. Illumination of solutions containing a thymine dimer and various oxidized and reduced flavins and deazaflavins revealed, that a reduced tetraacetylflavin can split a 1,3-dimethylthymine dimer substrate upon irradiation at λ = 300 nm. The authors report the necessity to perform the cleavage reaction at rather basic conditions (pH > 9). A more detailed investigation of the pH dependency of the cleavage reaction with reduced flavins as photo sensitizers was later reported by S. D. Rose and co-workers[64]: Irradiation of a solution containing reduced tetraacetylriboflavin, and dimethylthymine dimer, showed that the reduced flavin can initiate a cleavage chain reaction, giving rise to high quantum yields (up to ϕ = 1.3). This high quantum efficiency allowed the group to obtain a pH profile of the chain reaction. This study revealed half maximum dimer cleavage at pH $\approx$ 7.5 and maximum splitting efficiency at pH $\approx$ 8.5. Although both values are not in agreement with the pK_a value of the reduced flavin (pK_a = 6.5)[59], this investigation provided the first chemical evidence that the efficient dimer cleavage might require the *reduced flavin species* in its *deprotonated form*.[65]

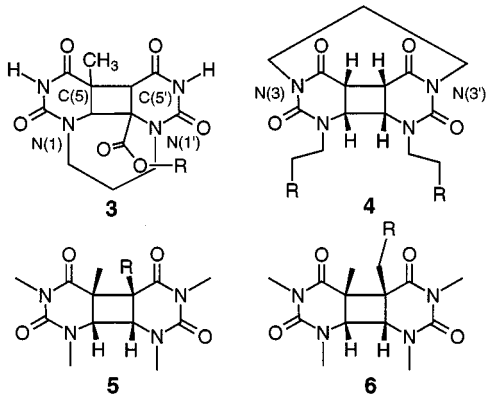
2.2 A Second Generation of Covalently Connected Model Compounds

In order to gain a deeper understanding of the dependencies of the flavin-mediated dimer cleavage, covalently linked model compounds are required, which enable a systematic investigation of the cleavage process. This information might then allow us to determine to which extent photolyases interfere with the cleavage reaction in order to achieve almost maximal quantum efficiency. Furthermore, model studies are required to deduce the function of specific structural and electronic properties of the photolyase cofactor and lesion-binding pockets.

For these investigations a series of highly variable covalently linked model compounds derived from **2** (see Fig. 4) were designed to mimic the quasi intramolecular enzymatic cleavage process. These compounds contain a bis(carboxymethyl)-functionalized cyclobutane-uracil (R = H) or -thymine (R = Me) dimer, which may possess either *cis-syn* (Figure 4), *trans-syn*, or *trans-anti* structure.[66] Depending on the question to be investigated, the model compounds contain a unique set of cofactors, that are covalently linked via amide bonds to the dimer unit. In this way a series of model compounds was prepared, which allowed the investigation of the cleavage process depending on external parameters such as the pH value or the medium polarity, or on internal parameters like the cofactor composition, the cofactor arrangement, or the dimer structure. The fact that all data are obtained with a structurally closely related set of model compounds, ensures maximum comparability of the results.

2.2.1 Preparation of the Photo Dimer Unit

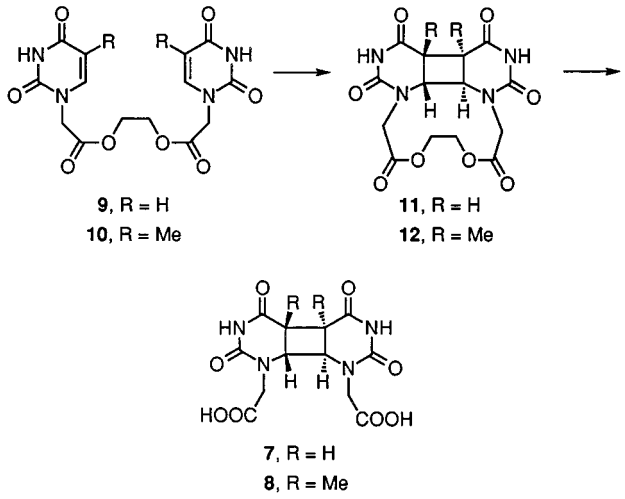
Today, most of the known DNA photolyase models, prepared predominantly by the groups of S. D. Rose[65][67], T. Begley[28], and C. Walsh,[68] contain a *cis-syn*-cyclobutane-pyrimidine dimer unit, like **3**[57][67] and **4**[28][68] which contain either a trimethylene bridge between N(1) and N(1′), or N(3) and N(3′). Such a bridge enforces the formation of the *cis-syn*-configured dimer during the synthesis and therefore eases the chemical preparation of photo dimers with *cis-syn* structure. Recent photo CIDNP studies showed, however, that in *N*(1),*N*(1′)-trimethylene-bridged dimers the initial cleavage of the C(5)−C(5′) bond is, in contrast to unbridged dimers like **5** and **6**, reversible.[69][70] In addition, the X-ray crystal structure of an *N*(3),*N*(3′)-trimethylene-bridged dimer reveals an almost planar cyclobutane ring, in contrast to the unbridged dimer substrate that possesses a pronounced cyclobutane ring twist of approximately −28° (CB⁻ pucker).[71] This twist is believed to be an important factor that determines the cleavage efficiency.[51][72][73] Both observations restrict the ability of bridged *cis-syn*-pyrimidine dimers to mimic the natural cleavage process. The own set restriction to avoid functionalization of the critical cyclobutane moiety and our goal to compare uracil dimers with thymine dimers excluded the alternative dimers **5**[74] and **6**[65][75] as lesions-mimicking building blocks for us as well.



Recently, P. G. Schultz and co-workers introduced the *trans-syn*-configured bis(carboxymethyl)-functionalized uracil and thymine dimers **7** and **8** as novel, unbridged photo-lesion mimics (Scheme 1).[76][77] These dimers were used for the generation of catalytic antibodies with dimer-splitting activities. Although the *trans-syn*-configured dimers are readily available, the synthetic strategy to irradiate the temporarily ethylene glycol bridged dimer precursors **9** and **10** to **11** and **12** fails for the preparation of the most desired *cis-syn*-configured compounds **13** and **14**.[78] This finding is in agreement with an observation of K. N. Ganesh and co-workers, who obtained only *trans-syn*-configured cyclobutane photo dimers upon irradiation of various *N*(1),*N*(1′)-polyoxyethylene-linked bis(thymine) and bis(uracil) compounds.[79]

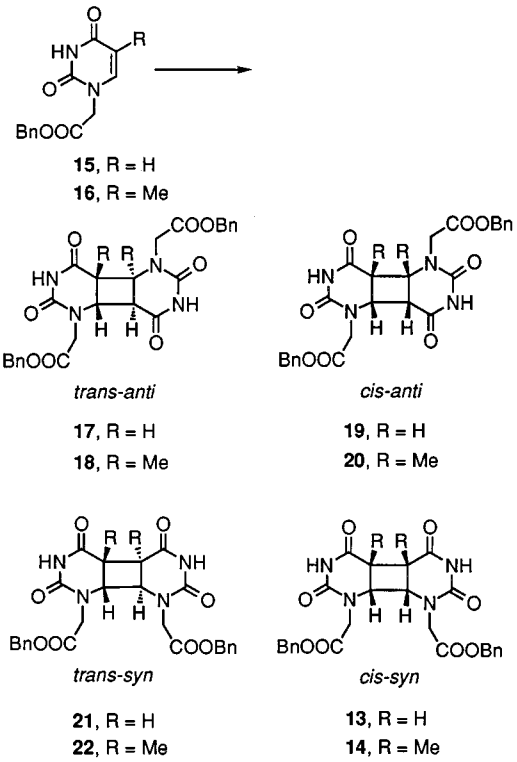
We found that the direct irradiation of benzyl ester protected carboxymethyluracil **15**[78] and carboxymethylthymine **16**[80] in pure acetone with a medium- pressure mercury

Scheme 1. Synthesis of bis(carboxymethyl)-functionalised *trans-syn*-cyclobutane-uracil and -thymine dimers[76]



lamp yields all four possible cyclobutane-uracil and -thymine dimers **13**, **14**, **17**−**22** in analytically pure form in one step (Scheme 2). All dimers can be separated by a combination of selective precipitation, chromatography and recrystallization. X-ray crystal-structure analyses of the *cis-syn*- and the *trans-syn*-configured uracil dimers **13** and **21** and of all four thymine dimers **14**, **18**, **20** and **22**, in combination with NMR-spectroscopic investigations allowed the unambiguous assignment of all four uracil and thymine di-

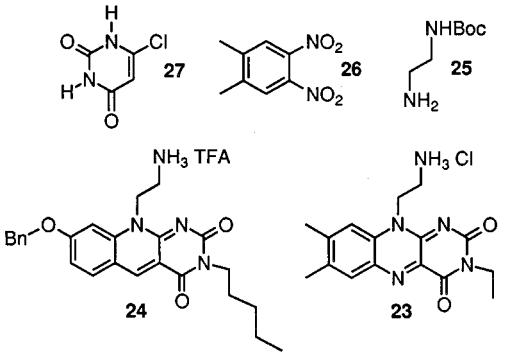
Scheme 2. Synthesis of bis(carboxymethyl)-functionalised *trans-syn*-, *trans-anti*-, *cis-anti*- and *cis-syn*-cyclobutane-uracil and -thymine photo dimers[78]



mer structures. The cleavage of the benzyl ester protecting groups was readily achieved using catalytic hydrogenation. This now allows the synthesis of most of the four uracil[78] and thymine[80] dimer dicarboxylic acids in gram quantities as required for the preparation of the desired flavin- and deazaflavin-functionalized model compounds.

2.2.2 Preparation of the Cofactor Units

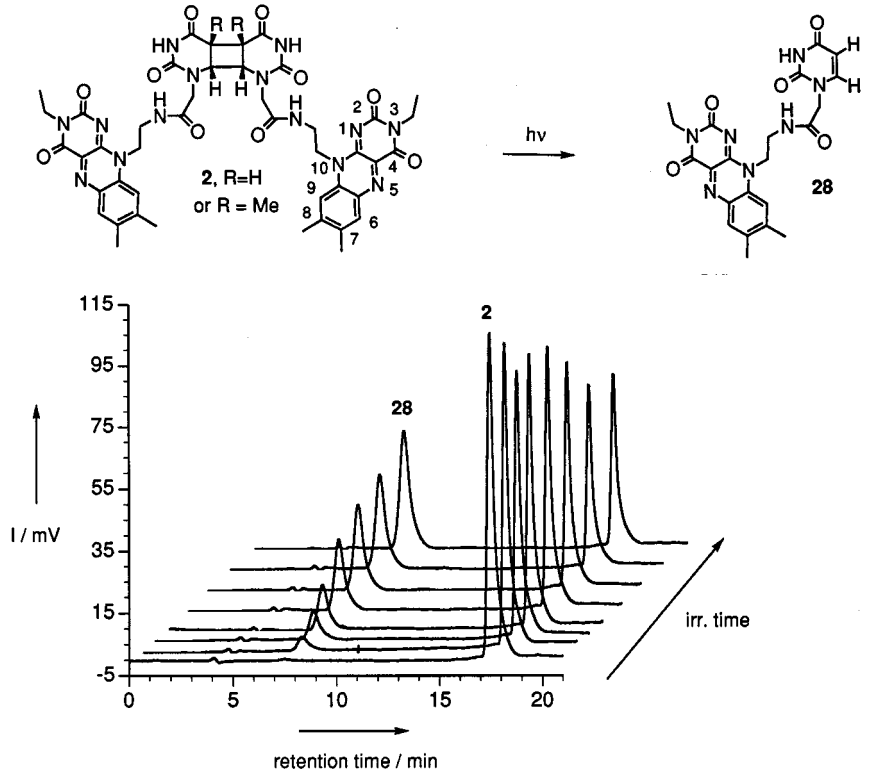
The required aminoethyl-functionalized flavin and deazaflavin cofactor moieties **23** and **24** were synthesized based on general flavin and deazaflavin syntheses procedures developed by R. Kuhn[81][82] and F. Yoneda[83] from *mono*-Boc-protected ethylenediamine **25**, 1,2-dinitro-4,5-dimethylbenzene (**26**)[84] and 6-chlorouracil (**27**)[85] after activation of the bis(carboxymethyl)-functionalized dimer building blocks with BOP and reaction with either **23** or **24**.[84]



2.3 General Photo Cleavage Properties

Initial light-induced cleavage experiments clarified the ability of these model compounds to mimic the enzymatic cleavage (repair) reaction. For the cleavage experiments model compounds like **2** were dissolved in various solvents in standard UV cuvettes and stoppered with a rubber septum. The solution required intensive deoxygenation prior to the reduction of the flavin units. Flavin reduction to $\mathbf{2H}_2^{2-}$ was performed by (1) the addition of a reducing agent like sodium dithionite, (2) photo reduction upon irradiation of the solution in the presence of an e^- donor like triethylamine or (3) hydrogenolytic reduction with H_2 and $Pd/BaSO_4$. Complete reduction of the flavin chromophore can be determined by UV and fluorescence spectroscopy before each measurement.[86] The reduced samples were stirred and irradiated with a monochromatic light beam. After certain time intervals, small aliquots were removed from the solution, immediately reoxidized by the admittance of oxygen and analyzed by reverse-phase HPLC. The series of HPL chromatograms (cleavage of **2** or **29** to **28**) depicted in Figure 4, underlines that the reaction of the model compound $\mathbf{2H}_2^{2-}$ to the photo-cleaved product **28** (after reoxidation) is a clean conversion, since no other products were detectable. The obtained peaks for the model compound and for the reaction product were integrated and normalized against the total peak area. This value was used to calculate the reaction yield at any given irradiation time. For the calculation of the quantum yields ϕ (ϕ = number of reacted molecules/number of absorbed photons) the intensity of the

Figure 4. Typical HPL chromatograms obtained during the splitting assay for **2** or **29** reacting to **28**



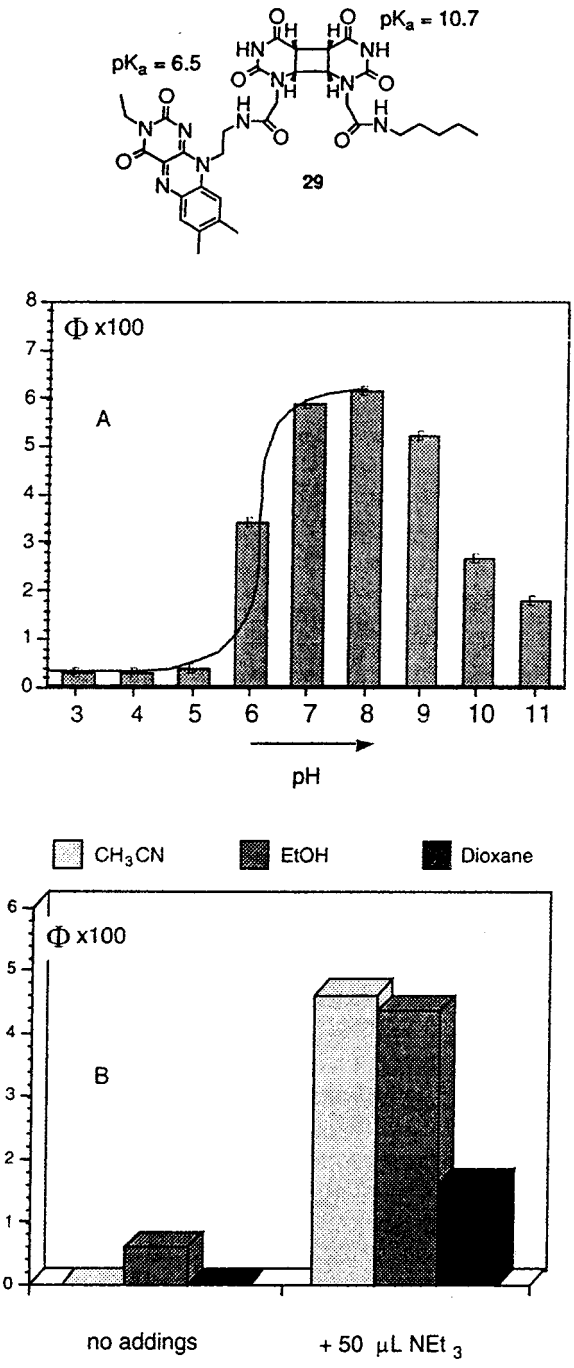
light beam was measured using ferrioxalate actinometry.[87] The observation that irradiation of a solution containing the photo dimer **13** and reduced flavin yielded no photo product under the same reaction conditions, even after prolonged irradiation, was taken as a strong evidence for the strict intramolecularity of the splitting process. No photo cleavage was observed in the absence of a reduced flavin or without prior reduction of the flavin chromophore within our model compounds, which proved the strict requirement for a covalently attached, reduced flavin moiety and showed that the *background splitting reaction under our conditions is close to zero.* No cleavage is observed in the absence of light. *All these measurements proved that these covalently linked compounds are the first models which are able to mimic the quasi intramolecular enzymatic cleavage reaction.* The clean light-induced conversion into the photo-split products and the negligibility of the background reaction made these models ideal candidates for the intended systematic investigation of the dependencies of the flavin-induced cleavage reaction.

2.4 Investigation of the pH Dependency of the Cleavage Reaction[84]

Investigation of the pH dependency of the splitting process was required in order to clarify how the deprotonation of the reduced flavin chromophore affects the cleavage reaction. The monoflavin-monopentylamide model compound **29** (Figure 5) was chosen for these experiments due to its superior solubility in organic and aqueous solvents. Figure 5A shows the quantum yields obtained for the photo cleavage of **29** at λ = 366 nm in water, buffered at various pH values. Very low cleavage activity is observed below pH = 6 and maximal splitting rates were measured above pH = 7. Intermediate rates were obtained between pH = 6 and 7. These data are in full agreement with the pK_a value of the reduced flavin (pK_a = 6.5).[59] and therefore support the view that the deprotonation of the reduced flavin is absolutely required for the efficient photo-induced splitting.[64] Further measurements in organic solvents (acetonitrile, ethanol and dioxane), using hydrogenolytic reduction, support this result. As depicted in Figure 5B, no cleavage is observed in the absence of base. Addition of triethylamine into the reaction mixture, however, caused upon irradiation the immediate cleavage of **29**, which shows again that the addition of base is strictly necessary for the photo reaction. Intensive UV-spectroscopic investigations of the model compound **29** in organic media in the absence and in the presence of triethylamine proved the presence of the protonated flavin species **29H**$_2$ directly after catalytic hydrogenation and in the absence of base. Addition of triethylamine caused, as expected, the deprotonation of the reduced flavin unit to **29H**$^-$.

Figure 6 summarizes the mechanistic conclusion derived from these results. Deprotonation of the reduced flavin species FlH$_2$ to FlH$^-$ increases the electron-donating capabilities of the flavin cofactor. The reduced flavin moiety FlH$_2$, however, as a masked phenylenediamine, should also be able to initiate the photo cleavage, if we consider that

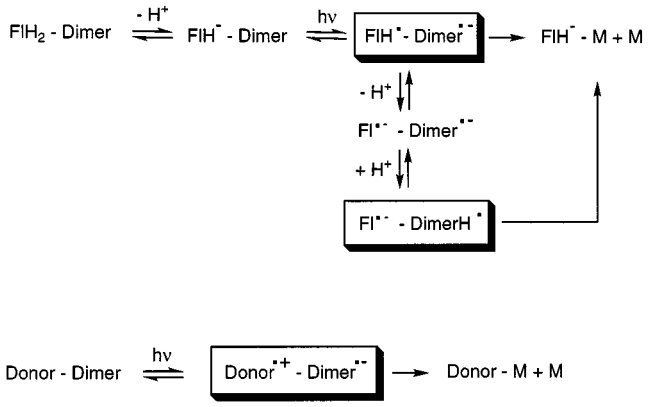
Figure 5. Investigation of the cleavage efficiency depending on the pH[84]



the photo cleavage can be initiated by various arylamine donors or indole derivatives. We therefore believe that the absolute requirement to deprotonate the flavin is not readily explained with the need to increase the electron richness of the electron donor alone, but must have additional mechanistic reasons.

One of the factors which influences the cleavage efficiency is the lifetime of the post-electron transfer intermediate Dimer$^{\bullet-}$[23][88]. Model flash photolysis investigations by D. E. Falvey[89] with dimethylaniline as the elec-

Figure 6. Schematic representation of the splitting mechanism

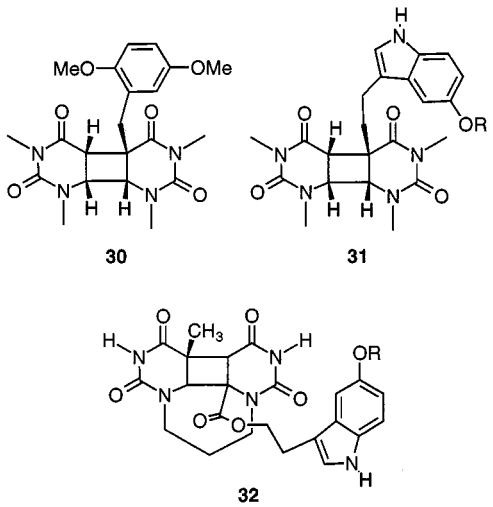


tron donors showed that the dimer cleavage proceeds on the microsecond time scale ($k \approx 10^6\ s^{-1}$). Electron transfer from a neutral electron donor, such as a reduced flavin (FlH_2) to the dimer would give a zwitterionic intermediate $Donor^{\bullet+}-Dimer^{\bullet-}$[90], which should possess a high driving force for charge recombination and therefore is assumed to yield a very short-lived intermediate. Photo CIDNP studies, performed by H. Fischer and co-workers, with a dimethylthymine dimer solution (acetonitrile), revealed that the dimer cleavage, if induced by a neutral donor (2-methoxyindole) is indeed not able to efficiently compete with the back electron transfer.[53] Electron transfer from a negatively charged electron donor, like a reduced and deprotonated flavin (FlH^-), however, would yield a non-zwitterionic intermediate $FlH^{\bullet}-Dimer^{\bullet-}$ in a simple charge-shift reaction. Such a negatively charged intermediate, should possess a much longer life time, consequently yielding to a more efficient splitting. In addition, the formed neutral flavin radical intermediate $FlH^{\bullet}$ possesses a significant stability, which is further increased if it is bound as the $FADH^{\bullet}$ to the photolyase apoenzyme. In fact, most of the isolated DNA photolyases contain the FAD unit in the blue radical form.[22] A reasonable explanation for the need to deprotonate the reduced flavin could therefore be to avoid a zwitterionic intermediate!

Based on the available experimental data we cannot exclude that the intermediate $FlH^{\bullet}-Dimer^{\bullet-}$ undergoes further protonation and deprotonation reactions in order to gain an even better stabilized intermediate. T. P. Begley suggested that the flavin radical might become deprotonated after the initial electron transfer due to its rather low pK_a ($pK_a = 6.5$).[59] This would generate a double negatively charged $Fl^{\bullet-}-Dimer^{\bullet-}$ intermediate,[28] which can undergo further dimer protonation to give an $Fl^{\bullet-}-DimerH^{\bullet}$ intermediate.

2.5 Investigation of the Solvent Dependency of the Cleavage Reaction[84]

In order to support the hypothesis of a negatively charged $FlH^{\bullet}-Dimer^{\bullet-}$ or $Fl^{\bullet-}-DimerH^{\bullet}$ intermediate, solvent dependent measurements were performed with the monoflavin–monopentylamide model compound **29**. Thus far, excellent investigations of the solvent dependency of the splitting reaction were performed by S. D. Rose and coworkers with Donor–Dimer model systems such as **30**–**32**.[65][74][75] These experiments revealed a rather strong solvent dependence of the cleavage rate, with high cleavage rates ($\phi = 0.3$) obtained in the least polar solvent mixtures (isopentane/dioxane, 99:1). Splitting efficiencies measured in polar solvents like water were 25 times lower ($\phi = 0.02$). This surprising result was explained with the need to suppress a fast and unproductive charge recombination process in the zwitterionic intermediate. Since unpolar media strongly destabilize such a zwitterion, S. D. Rose suggested that this destabilization might increase the $\Delta G^{\neq}$ for the charge recombination process so much, that the back electron transfer is shifted energetically into the Marcus inverted region, where it would be significantly slowed down.[11]



Two sets of experiments were performed in order to clarify the solvent effect with flavin-containing model compounds. In the first set of experiments the splitting rates were measured with compound **2** and **29** in water/ethanol and water/ethylene glycol mixtures (Table 2). The most efficient cleavage was observed in pure water with $\phi = 0.06$. Addition of ethanol or ethylene glycol reduced the cleavage efficiencies by a factor of not more then 2 to $\phi = 0.03$. Measurements in various organic solvents (Table 1), using the catalytic reduction methodology, support this result.

Table 1. Solvent dependence of the quantum yield Φ for compound **29**

Solvent	ε_r[a]	Φ[b]
1,4-dioxane	2.21	0.016
benzene	2.27	–[c]
ethyl acetate	6.02	0.024
tert-butyl alcohol	12.47	0.037
ethanol	24.55	0.044
methanol	32.66	0.040
acetonitrile	35.94	0.046

[a] Relative permittivity (dielectric constant) for the pure liquid at 25°C. – [b] Quantum yields of model compound **29**. – [c] No quantum yield detectable even after prolonged irradiation.

The best cleavage efficiencies were obtained in the most polar solvents, methanol, ethanol and acetonitrile ($\phi = 0.04$). In apolar solvents like dioxane or ethyl acetate ($\phi = 0.02$) the cleavage rate is approximately twice as slow.[84]

Table 2. Solvent dependence of the quantum yield Φ for compounds **2** and **29**

Solvent mixture (% etgl)[a]	Φ (**29**)	Φ (**2**)
water/etgl (0)	0.062	–[b]
water/etgl (33)	0.052	–[b]
water/etgl (50)	0.051	0.049
water/etgl (66)	0.041	0.047
water/etgl (100)	0.033	0.038
DMF	0.051	0.045

[a] etgl: ethylene glycol. – [b] No quantum yield detectable due to low solubility.

Both experiments showed increased splitting efficiencies in polar solvents and a rather low total solvent dependence. This underlines that in flavin-containing model compounds such as **2** and **29** reaction intermediates are formed, which are not comparable with the zwitterionic intermediates postulated for neutral Dimer–Donor systems. The obtained medium-dependent data are in full agreement with non-zwitterionic reaction intermediate(s) and therefore support the postulated charge shift process.

Figure 7. Depiction of some of the critical side chains which form the FAD-binding pocket in the DNA photolyase from *E. coli*[29]

A close inspection of the FAD-binding pockets in type-I photolyases from *E. coli* and type-II photolyases from *A. nidulans* leads to the interesting observation, that both enzymes bind the FAD cofactor in a highly conserved, rather polar environment (Figure 7).[29] In the *E. coli* enzyme, the flavin is positioned in van der Waals contact to a salt bridge formed by Arg344 and Asp372 as depicted in Figure 7. The FAD is involved in hydrogen bonding via O(2) to Glu274 (water-mediated) and via O(4) to the backbone amide of Asp374. The N(5) of the FAD is in close proximity to the side chain oxygen atom of Asn378. In the reduced cofactor status, the N(5)···O contact might contribute to the stability of the $FADH^{\bullet}$ radical and could be one of the essential features of the binding pocket required for the stabilization of the $FADH^{\bullet}$ radical intermediate.[91] The N(1) of the FAD is hydrogen-bonded to its own 3′-OH group, which was suggested to stabilize the negative charge of the $FADH^{-}$[29]. Many polar amino acid side chains like those of Arg226, Asn341 and Arg342 surround the FAD and contribute to the polarity of the binding pocket. Furthermore, the FAD is solvent-accessible through a hole in the protein, which is the putative dimer lesion binding side. All these interactions are highly conserved. As partially depicted in Figure 8, the *A. nidulans* FAD-binding pocket contains also a salt bridge (Arg352 Asp380) in van der Waals contact to the flavin and features an identical set of amino acid side chains around the FAD, with the N(1) of the FAD hydrogen-bonded to the 3′-OH group and the N(5) in close proximity to the Asn386.

3. The Function of the Second Cofactor

3.1 General Considerations

Today, all well-studied DNA photolyases contain, in addition to the crucial FAD, either a methenyltetrahydrofolate (MTHF) or an 8-hydroxy-5-deazaflavin (8-HDF)[22] as a second cofactor. Kinetic investigations of photolyases lacking this second cofactor revealed that they still possess repair activity but: (1) The second cofactor increases the repair efficiency at a given irradiation intensity and (2) the second cofactor shifts the wavelength at which maximal repair occurs from 370 nm in "flavin-only" photolyases to 400 nm in MTHF- and even to 430 nm in 8-HDF-containing enzymes.[92] Replacement of the FAD with an dFAD yields a catalytically incompetent enzyme (irradiation $\lambda > 350$ nm).[93] This result shows that under physiologically relevant conditions, a dFAD is not able to drive the electron transfer based repair process. Some remaining repair activity of dFAD photolyases was, however, obtained upon irradiation at $\lambda < 350$ nm which could be due to either the excitation of a tryptophane residue in the vicinity of the dimer binding side or indicates that a reduced dFAD also exhibits some rudimentary cleavage activity at these shorter wavelengths.[94] All these experiments, together with results from steady-state UV and fluorescence experiments lead to the conclusion that the second cofactor is not involved in electron transfer processes but functions entirely as a photo antenna. The second cofactor therefore has a function

closely related to antenna pigments in the photosynthetic reaction center, which also funnel excitation energy to the primary special pair electron donor.[95]

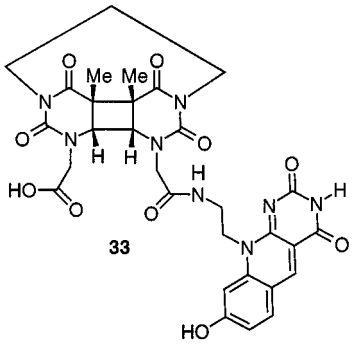
The antenna function requires an intensive interaction of the MTHF or the 8-HDF with the corresponding FAD suggesting a close arrangement of both cofactors within the enzyme. The center-to-center distance between both cofactor units was, however, found to be surprisingly large with 16.8 A in the *E. coli* (MTHF, FAD) enzyme[29] and 17.5 A in the *A. nidulans* (8-HDF, FAD) protein (see Figures 2 and 8).[30] Time-resolved fluorescence investigations of the *A. nidulans* enzymes by P. F. Heelis and co-workers showed that despite the large cofactor separation, the energy transfer proceeds rapidly ($k_{EET} = 1.9 \times 10^{10}$ s^{-1}) and with high efficiency (98%) between the 8-HDF and the FAD in the *A. nidulans* protein.[96] The reason is a favorable orientation of the transition dipole moments. The X-ray crystal structure revealed an angle between the transition dipoles of both cofactors of 35.6°. This creates a favorable orientation factor of $\kappa^2 = 1.6$ and an efficient energy transfer based on the Förster theory for long-range dipole-dipole interactions.[97][98] In the MTHF-containing *E. coli* enzyme, the energy transfer is far from optimal with a transfer rate of $k_{EET} = 4.6 \times 10^9$ s^{-1} and a low efficiency of only 62%. To a minor extent, this is due to the large cofactor separation. Most unfavorable is the orientation of their transition dipole moments being almost perpendicular to each other. This produces an orientation factor κ^2 close to zero.[30][99] In order to explain this low value, it was suggested that the energy transfer in photolyases is not rate-determining and was not optimized during evolution. In the photosynthetic process, however, the observation that the energy transfer from the last antenna pigment to the primary electron donor is slow, due to a large distance between the final antenna and the electron donor, led to the proposal that the system needs to avoid an unproductive electron transfer from the donor to the antenna.[100] With a redox potential of the reduced and light-excited flavin of about $E_{red}^* = -2.8$ V[101] and of the dimer $E_{red} = -2.2$ V[101] an electron transfer to a deazaflavin with a redox potential E_{red} around -1.5 V[102] seems at a first glance to be thermodynamically feasible.

A close inspection of the UV and fluorescence spectra of the *A. nidulans* photolyase revealed that type-II DNA photolyases use the deprotonated version of the 8-hydroxy-5-deazaflavin cofactor ($pK_a = 6$) as the light-gathering cofactor. Most of the today known 8-hydroxy-5-deazaflavin-containing enzymes, however, require the deazaflavin in its protonated form (absorption maxima at 390 and 400 nm) as a low-potential hydride transfer agent.[103] The deprotonated 8-O^--deazaflavin is, in contrast, rather redox-incompetent and features an absorption maximum at $\lambda = 430$ nm.[68] In order to ensure deprotonation of the 8-HDF within the enzymatic binding pocket, the enzyme has to provide basic side chains in the vicinity to the 8-OH group of the 8-HDF. Inspection of the X-ray crystal structure of the photolyase from *A. nidulans* (Figure 8) shows the two basic side chains of Lys248 and Arg51 at the bottom of the 8-HDF binding pocket in close proximity to the 8-OH group.[30] Both residues are highly conserved and may be responsible for the 8-HDF deprotonation.

Figure 8. Depiction of some of the critical side chains which form the 8-HDF- and FAD-binding pocket in the DNA photolyase from *A. nidulans*[30]

3.2 Deazaflavin-Containing Model Compounds

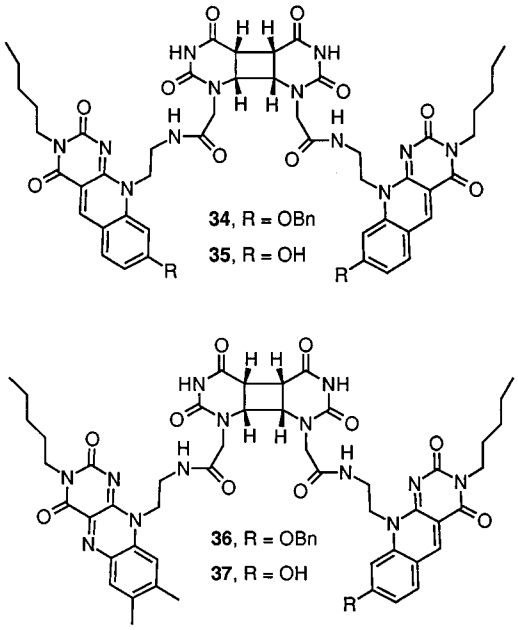
An early investigation of whether deazaflavins are able to drive the electron transfer based repair was performed by A. P. M. Eker with solutions of 5-deazaflavin and thymine dimers. These experiments revealed that oxidized deazaflavins are able to cleave pyrimidine dimers to a small extent upon irradiation.[104] This result was confirmed by C. Walsh and co-workers with the covalent model compound **33** in which a 8-hydroxy-5-deazaflavin is covalently linked to an *N*3,*N*3′-trimethylene-bridged thymine dimer. Irradiation of the model compound **33** at $\lambda = 436$ nm at pH = 11.5 yields dimer cleavage. The required high pH and the very low quantum yields ($\phi < 10^{-4}$), however, made an electron transfer role of the second cofactor during the enzymatic repair process, based on these model studies, highly unlikely.[68]



In order to perform a systematic study of the ability of deazaflavins to influence the dimer cleavage, depending on its oxidation and protonation state, a series of deazaflavin-containing model compounds **34**−**37** depicted in Figure 9 was prepared.[85] The first model compounds **34** and **35** contain a *cis-syn*-uracil dimer and two deazaflavin units covalently attached to the dimer. The deazaflavins possess either a benzylated 8-OH group (OH form) or a debenzylated and deprotonated 8-OH group (O^- form). Irradiation of both model compounds **34** and **35** at various wavelengths and subsequent HPLC analysis of the assay solutions showed that both compounds were completely stable upon irradiation. No photo splitting into the expected photo cleavage products was detected, which shows that both deazaflavins are unable to perform the cleavage reaction under those conditions. Reduction of the benzylated deazaflavin in model compound **34** to $\mathbf{34H_4}$ and irradiation at $\lambda > 350$ nm gave also no photo product, which indicates that at these wavelengths also the reduced deazaflavin (reduced OH form) is ineffective.

The two mixed model compounds **36** and **37** were prepared in order to study how the presence of the deazaflavins affects the flavin-driven cleavage process. Steady-state fluorescence studies show that the deazaflavin fluorescence in the mixed model compounds **36** and **37** is strongly quenched by the adjacent flavin chromophore, indicating an efficient energy transfer from the deazaflavins to the flavin units. Selective reduction of the flavin unit in both model systems was performed in order to simulate the active co-

Figure 9. Deazaflavin-containing model compounds **34**−**37** for the investigation of intercofactor energy transfer reactions[85]

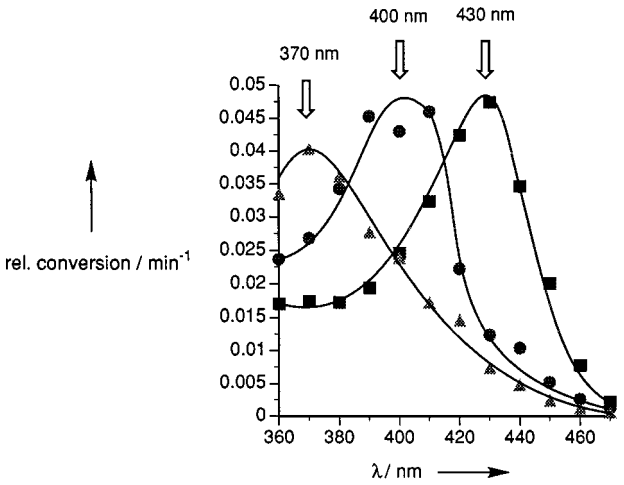


factor status in type-II DNA photolyases. Photo reduction of the flavin in model compound **36** in the presence of triethylamine afforded the semi-reduced model compound $\mathbf{36H^-}$.[105] In compound **37**, reduction of the flavin unit to $\mathbf{37H^{2-}}$ is possible with sodium dithionite. Prior to each irradiation experiment the cofactor status in the model compounds was unequivocally confirmed by UV and fluorescence spectroscopy.

The action spectra depicted in Figure 10 show the splitting rates depending on the irradiation wavelength for both semi-reduced model compounds $\mathbf{36H^-}$ and $\mathbf{37H^{2-}}$ in comparison to the "flavin-only" model compound $\mathbf{29H^-}$. From the maxima in the action spectra it is evident that both types of deazaflavins are able to transfer excitation energy to the reduced and deprotonated flavin (FlH^-) species. In model compound $\mathbf{29H^-}$ maximal repair occurs at 370 nm as expected for a "flavin-only" containing model compound. This maximum is shifted to 400 nm in the presence of a deazaflavin in its OH form (model compound $\mathbf{36H^-}$) and to 430 nm in $\mathbf{37H^{2-}}$, which contains a deprotonated deazaflavin (O^- form). The maxima in the action spectra are in excellent agreement with the absorption maxima of the corresponding deazaflavin species, which proves that the energy for the splitting process is initially absorbed by the oxidized deazaflavin chromophores and subsequently transferred to the reduced flavin. Since the action spectrum of $\mathbf{37H^{2-}}$ is in full agreement with the *A. nidulans* spectrum,[92] the experiments support the presence and the light-gathering function of the deprotonated 8-HDF in *A. nidulans* photolyases. Since all MTHF-containing photolyases feature a maximum in their action spectra at 400 nm, the action spectrum of model compound **36** shows that the protonated deazaflavin would in principle be able to fulfill a light-harvesting function as well. The model studies, there-

fore, raise the interesting question why photolyases prefer to keep the 8-HDF cofactor in its deprotonated form.

Figure 10. Action spectra measured with the model compounds **29** (▲), **36** (●) and **37** (■)[85]



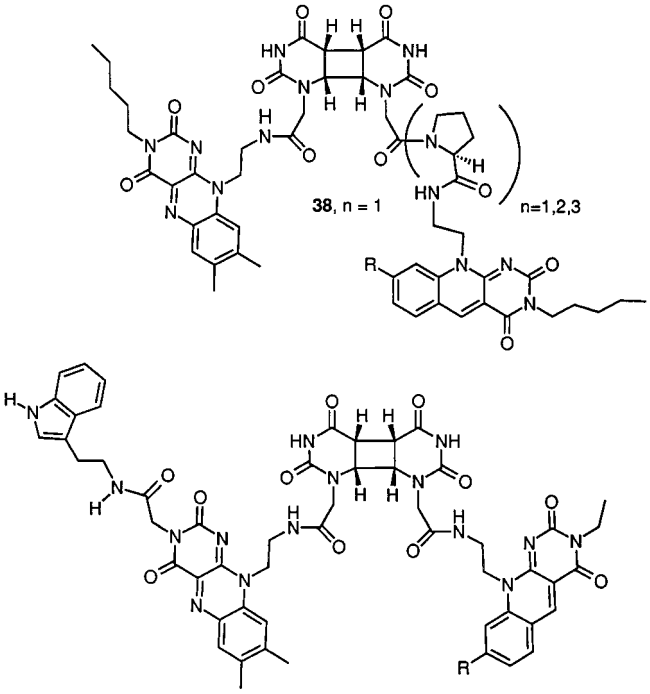
Although both model compounds **36** and **37** are able to simulate the efficient energy transfer, all three compounds **29**, **36** and **37** exhibit only comparable cleavage rates at their maximum repair wavelength. So far, all measurements were performed at rather high concentration $c = 5 \times 10^{-5}$ M, which yields solutions with a high absorptivity (extinction > 1). Upon irradiation, less photons are consequently directly absorbed by the reduced flavin, which decreases the splitting efficiency in the mixed flavin–deazaflavin model systems. Although initial concentration-dependent measurements show that this depression effect is rather small, further measurements at very low concentrations (extinction < 0.1) are now in progress to evaluate its magnitude in order to precisely determine to which extent the deazaflavins influence the splitting efficiency in our model compounds.

4. Outlook and Conclusions

In order to address the extent to which the splitting efficiency is affected by the cofactor–cofactor distance, the synthesis of a new series of model compounds derived from **38** (Figure 11) has been completed.[80] In these model compounds one to three proline units act as rigid spacers between the deazaflavin and the dimer unit to ensure a larger flavin–deazaflavin and deazaflavin dimer distance and to inhibit potential stacking interactions. Fluorescence measurements show that the energy transfer from the deazaflavin to the flavin still occurs and splitting measurement are now in progress to evaluate the repair rates depending on the irradiation wavelength and the cofactor–cofactor distance.

Future model studies should also address the question of how the reduction of the FAD is accomplished. It is currently believed that the reduction requires an electron transfer from a tryptophane residue to the light-excited FAD radical. In order to study this electron transfer possibility, model compounds such as **39** are currently being systematically evaluated. These model compounds contain the full

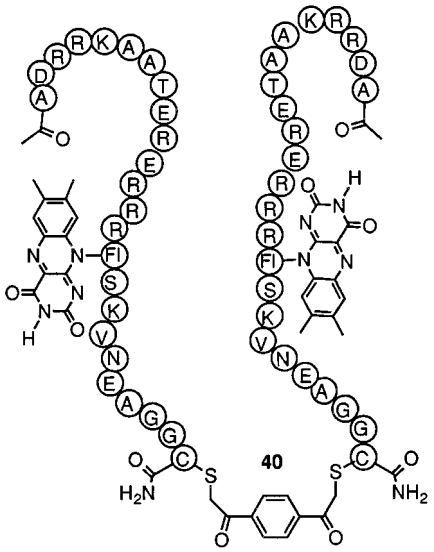
Figure 11. Representation of model compounds **38** and **39** prepared for the investigation of how the cofactor–cofactor separation effects the splitting reaction and to determine how the flavin reduction and the splitting reaction is influenced in the presence of a tryptophane unit

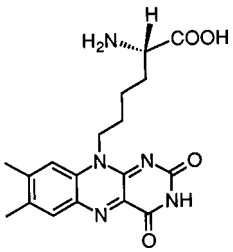


set of cofactors thought to be required for the repair reaction.[80] Initial measurements of model compound **39** show that the flavin fluorescence is strongly quenched in the presence of the electron-rich tryptophane residue.[106] Reduction of the flavin unit with sodium dithionite could, however, be achieved and initial splitting experiments show that despite the reduced fluorescence, **39** (R = OBn) cleaves to the expected photo products upon irradiation.

We hope that these more peptide-like model compounds will allow us to learn more about the parameters that determine the efficient repair of DNA UV-lesions by DNA photolyase enzymes. This information, together with the ability to prepare flavin- and deazaflavin-containing oligopeptides may then allow the preparation of small molecules featuring DNA photolyase activity. Very successful repair studies with tryptophane-containing peptides, performed by C. Helene and co-worker[107], show that the artificial repair is in principal possible. The group of J. K. Barton recently achieved the repair of thymine dimers within double-stranded DNA in an oxidative process, with the help of electron-abstracting metal complexes.[108][109] Our own experiments show that DNA-binding oligopeptides like **40** containing the flavin amino acid *L*-**41** incorporated are able to repair pyrimidine dimers cleanly in single-stranded DNA. All these achievements make us currently optimistic that the goal to develop small-molecule artificial DNA photolyases can be reached.[110]

Figure 12. Depiction of the cofactor peptide **40** containing the flavin amino acid *L*-**41** incorporated; the cofactor peptide **40** possesses the sequence of the DNA-binding domain of the transcription factor MyoD and was shown to repair a dimer-containing DNA single strand[110]





L-**41**

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