Investigation of Flavin-Containing DNA-Repair Model Compounds

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Abstract: Irradiation of DNA with UV-B light causes the formation of mutagenic DNA lesions such as *cis−syn* and *trans−syn* cyclobutane pyrimidine dimers. DNA photolyases are flavin-dependent repair enzymes which directly revert the mutagenic *cis−syn* pyrimidine dimers into the corresponding monomers by a light-facilitated repair reaction. To gain deeper insight into the repair process, we recently prepared flavin-containing model compounds which are able to mimic the repair reaction (Carell, T.; Epple, R.; Gramlich, V. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 620−623). This publication now contains a detailed description of the synthesis of a series of related model compounds and a comprehensive investigation of their cleavage properties. The results obtained help to unravel the requirements necessary for an efficient, flavin-mediated cleavage of pyrimidine dimers and provide insight into the factors on which the enzymatic repair process depends. The investigation of the cleavage reaction with *cis−syn*, *trans−syn*, and *trans−anti* cyclobutane pyrimidine dimer model compounds reveal an enhanced vulnerability for the *cis−syn* isomer. The *trans−syn* dimer is 10 times more stable. These results are comparable to those observed in a recent study on the *E. coli* enzyme. The excellent solubility of some of the model compounds has allowed a medium-dependent investigation of the flavin-initiated cleavage reaction. Increased cleavage efficiencies are observed in polar solvents such as water ($\phi = 0.06$) and acetonitrile ($\phi = 0.05$). The quantum yields decrease by a factor of 4 in solvents with very low polarity such as dioxane ($\phi = 0.01$). These results are not in agreement with earlier solvent-dependent evaluations performed with non-flavin-containing model compounds (Hartzfeld, D. G.; Rose, S. D. *J. Am. Chem. Soc.* **1993**, *115*, 850−854). The results, however, suggest that the unusually polar flavin-binding pocket, observed in the X-ray crystal structure of the *E. coli.* photolyase, might be required to increase the catalytic repair efficiency. Investigations of the cleavage reaction in the presence of acid and base in organic solvents emphasize the strict requirement for a deprotonated reduced riboflavin chromophore. The determined pH values for half-maximal (pH = 6.5) and maximal ($7 \leq \text{pH} \leq 9$) cleavage efficiencies are in agreement with the pK_a value (pK_a = 6.3) of the reduced riboflavin and reveal that physiological conditions are required to reach maximum catalytic cleavage efficiency.

Introduction

The irradiation of cells with UV-B light (280−320 nm) causes the formation of *cis−syn* cyclobutane pyrimidine dimers as the major photoproducts present in irradiated DNA.[1,2] The structurally related, highly mutagenic *trans−syn* lesions are formed to a much smaller extent, predominantly upon irradiation of single-stranded DNA.[2,3] Both DNA lesions are involved in the development of various skin cancers such as basal cell and squamous cell carcinomas and melanomas.[4−6] The observed depletion of the ozone layer causes an increasing UV-B radiation level and therefore a higher risk of skin cancer.[7,8] This scenario fuels research interests to elucidate the mechanisms developed by nature to remove the UV-induced photolesions from the genome.[2] DNA photolyases are flavin-containing repair enzymes which catalyze the efficient repair of the *cis−syn* cyclobutane pyrimidine dimers.[9] Elegant studies of various photolyases[2,9,10] have provided the currently accepted mechanism of the repair process. The photolyase-catalyzed monomerization of pyrimidine dimers requires sunlight which initiates an electron-transfer process from a reduced flavin cofactor in the enzyme to the DNA damage.[9,11,12] Spontaneous monomerization of the resulting radical anion and transfer of the surplus electron back to the riboflavin cofactor concludes the catalytic cycle.

Although the X-ray structure of the *E. coli* photolyase[13] gives a detailed picture of the cofactor arrangement and its protein environment, several important elements of the enzymatic process remain unsolved: (1) It is not currently known how the enzyme mediates the excited-state electron-transfer processes

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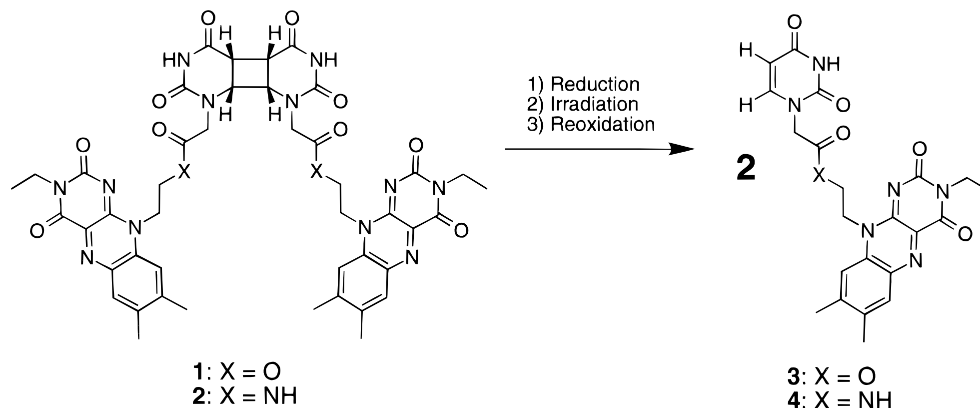

Figure 1. Representation of the electron-transfer-based cycloreversion of the model compounds **1** and **2**. Reaction products are the monomers **3** and **4**. The electron donor is a reduced flavin. The parent compounds **1** and **2** mimic the light-driven DNA-repair process catalyzed by the enzyme DNA photolyase.

in order to achieve almost quantitative repair efficiency (ϕ = 0.6), and (2) it remains mysterious why photolyases are unable to recognize and efficiently repair the minor but highly mutagenic *trans*−*syn* photolesion.[14] This *trans*−*syn* isomer poses a serious mutagenic threat, especially in organisms which possess a single-stranded DNA genome during their life cycle.[15,16]

So far, the investigation of the dependencies of the repair reaction with flavin model systems were limited to the analysis of mixed solutions containing flavin derivatives and cyclobutane dimer models.[17−20] These studies were hampered by low quantum yields of the photochemical cycloreversion process (ϕ = 10^{-3}−10^{-4}) and by the limited solubility of the reaction partners in solvents other than water. Recent solution studies, performed by S. D. Rose and co-workers, in which flavins initiate a cleavage chain reaction, have shown that deprotonation of the reduced flavin is a strict requirement.[20] This investigation provided estimates of the pH values required for half-maximal (pH ≈ 7.5) and maximal (pH ≈ 8.5) repair efficiency.[20] These high values, however, are not in agreement with the pK_a of the reduced riboflavin (pK_a = 6.3),[21] and they raise the question of how photolyases achieve maximal repair efficiency under physiological conditions (pH = 7).

The cleavage rates of *cis*−*syn*, *trans*−*syn*, and *trans*−*anti* cyclobutane dimers were measured—using nonflavin-containing model systems—to investigate potential cleavage rate differences. The results, however, were inconclusive. Higher cleavage rates for *trans*−*syn* isomers were determined in experiments performed with anthraquinone,[22] *p*-chloranil, and 9,10-dicyanoanthracene sensitizers.[23,24] In contrast, less efficient cleavage of the *trans*−*syn* isomers was reported with phenanthrene and *p*-dicyanobenzene sensitizers[25] and in studies using γ-radiolysis.[26]

We have recently communicated the preparation of covalently linked flavin-containing model compounds.[27] Initial cleavage experiments indicated a higher vulnerability of the *cis*−*syn* isomer in the flavin-mediated monomerization process in comparison to the *trans*−*syn* and the *trans*−*anti* isomer. We now report the synthesis of a series of related model compounds, derived from the parent compounds **1**[27] and **2**, which cleave photoinduced into the photosplit products **3** and **4** as depicted in Figure 1. An HPLC-based assay was developed, which allows quantification of the cleavage rates and determination of the quantum yields. The most active compounds feature quantum yields of up to ϕ = 10^{-1}−10^{-2}, which is 2−3 orders of magnitude higher than previously observed with flavin-containing solutions.[17−19] The presented investigation of the monomerization reaction shows that the flavin-induced repair reaction is weakly solvent dependent and most efficient in solvents possessing a high dielectric constant. This is in contrast to earlier observations made with non-flavin-containing model compounds.[28−31] Our results, however, suggest an explanation for the unusually polar flavin-binding pocket observed in the X-ray structure of the *E. coli* enzyme.[13] pH-dependent measurements, performed with more stable amide-linked model compounds show maximal cleavage efficiency at 7 pH ≤ 9, in agreement with the known pK_a values of the reduced riboflavin and of the cyclobutane uridine dimer lesion. This result helps

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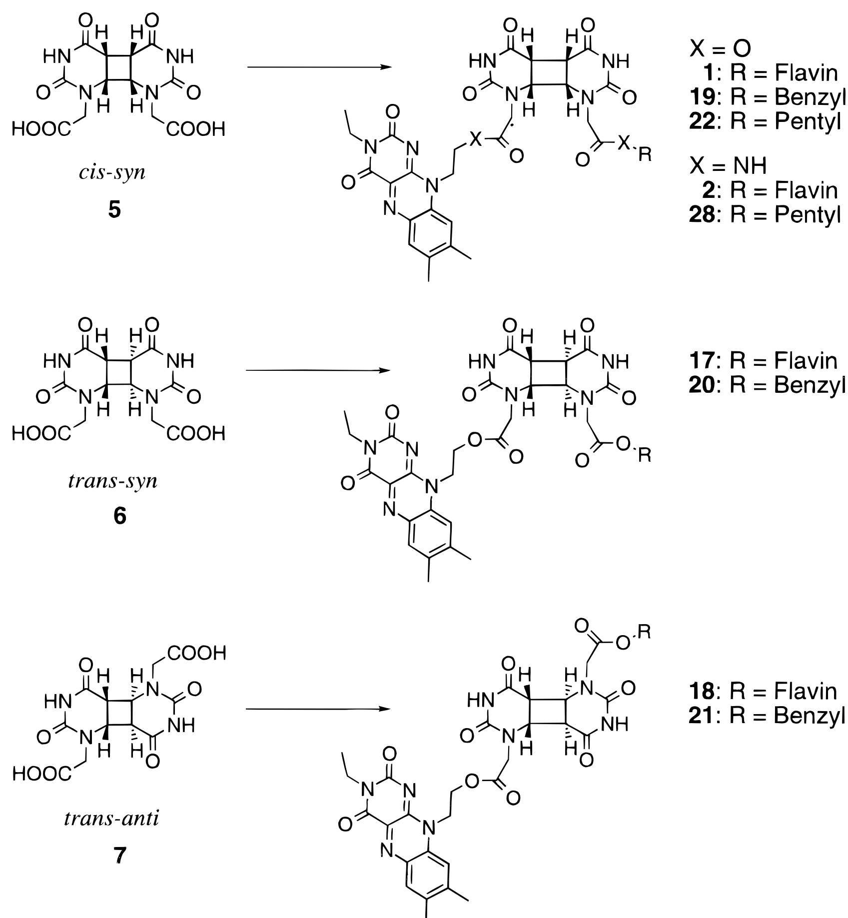
Scheme 1. Model Compounds for the Flavin-Initiated Cycloreversion of Pyrimidine Dimers by a Light-Driven Electron-Transfer Process[a]



[a] All compounds were prepared from the *cis*−*syn*, *trans*−*syn,* and *trans*−*anti* cyclobutane uracil dimers **5**−**7**[17] and the flavin alcohol **8** and the flavin amine **9**.

to clarify how photolyases achieve nearly quantitative repair efficiency (ϕ > 0.6) under physiological conditions.

Finally, more detailed measurements of the cleavage properties of various cyclobutane uracil dimer isomers, incorporated into the model compounds, allowed quantification of the increased cleavage rate for the *cis*−*syn* pyrimidine dimer.

Results and Discussion

Design and Preparation of the Model Compounds. The model compounds presented in Scheme 1 were designed as previously reported.[27] As the lesion component, a *cis*−*syn*, a *trans*−*syn,* and a *trans*−*anti* N^1,$N^{1\prime}$-dicarboxymethyl cyclobutane uracil dimer was covalently attached to one or two flavin chromophores. The three pyrimidine dimer dicarboxylic acids **5**−**7** were prepared as described. The structures of the three isomers were confirmed by X-ray crystal structure analysis for the *cis*−*syn* and the *trans*−*syn* isomers **5** and **6**.[27,32,33]

The synthesis of the hydroxyethyl and aminoethyl 6,7-dimethylflavin chromophores **8** and **9**, required for the synthesis of the model compounds, are depicted in Scheme 2. The preparation of the hydroxyethyl flavin was recently communicated.[27] The synthesis includes protection of the amino group in **10** with trifluoroacetic acid anhydride.[34] Reaction of **11** with 1-bromo-2-methoxyethane (**12**)[35] afforded **13**, which was hydrogenated to **14**. The flavin synthesis developed by R. Kuhn and co-workers[36] afforded the methoxyethyl flavin **15**. The final compound **8** was obtained after ethylation to **16** and cleavage of the methoxy protection group. Treatment of **8** with the pyrimidine dimer dicarboxylic acids **5**−**7** after in situ BOP[37] activation of the carboxylic acids and subsequent chromatography yielded the three bisflavin model compounds **1, 17,** and **18**.[27] To eliminate potential intramolecular flavin−flavin π-stacking interactions[38] which might obscure the quantum yield measurements, preparation of the monoflavin-substituted model compounds **19**−**22** was required. These compounds were synthesized by reaction of the corresponding activated pyrimidine dimer dicarboxylic acids **5**−**7** with 1 equiv of the hydroxyethyl flavin **8** and the subsequent addition of a large excess of benzyl alcohol (or pentyl alcohol) to the reaction mixture.

The synthesis of the aminoethyl-substituted flavin **9** (Scheme 2) was required for the preparation of amide-linked model

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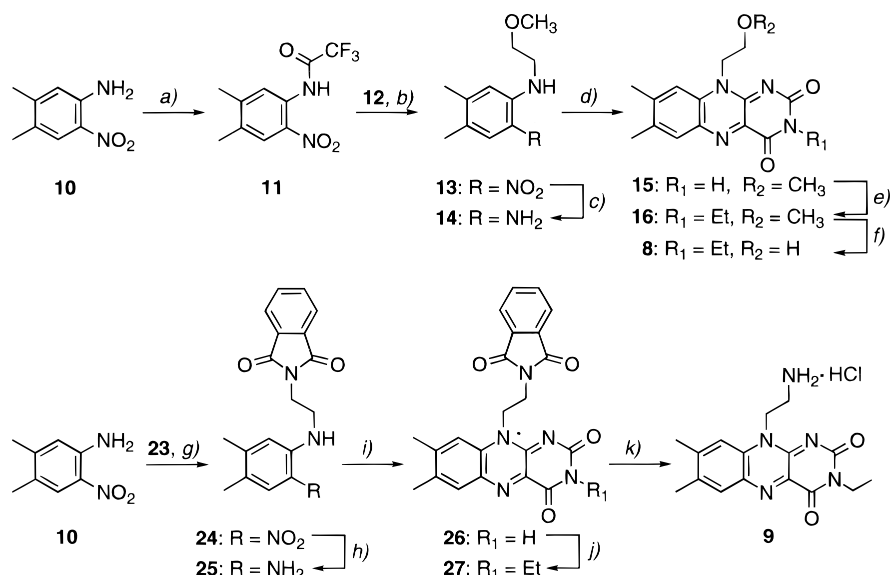
Scheme 2[a]



[a](*a*) $(CF_3CO)_2O$, room temperature (rt), 90%; (*b*) 1-bromo-2-methoxyethane[35] (**12**), K_2CO_3, DMF, 100 °C, 89%; (*c*) H_2, Pd/C, HOAc, rt; (*d*) HOAc, boric acid, alloxane monohydrate, (*e*) EtI, K_2CO_3, DMF, rt, 83%; (*f*) BBr_3, CH_2Cl_2, 0 °C, 81%; (*g*) 1.7 equiv of 1-(bromoethyl)phthalimide (**23**), 175 °C, 23.5%; (*h*) H_2, Pd/C, HOAc, 50 °C; (*i*) alloxane monohydrate, $B(OH)_3$, HOAc, rt, 47%; (*j*) EtI, K_2CO_3, DMF, rt, 86%; (*k*) concentrated HCl, reflux, 92%.

compounds required for measurements at high pH values. Reaction of **10** with (1-bromoethyl)phthalimide (**23**) afforded the phthalimido anilide **24**. Hydrogenation of **24** in acetic acid and treatment of the resulting aniline derivative **25** with alloxane and boric acid in acetic acid yielded the flavin derivative **26**, which was ethylated at N(3) with ethyl iodide to give **27**. Due to the instability of the isoalloxazine of phthalimide **27** in the presence of hydrazine,[39] we cleaved the phthalimide moiety by refluxing compound **27** in concentrated HCl and isolated the target compound **9** as its stable hydrochloride salt. The flavin ethylamine **9** could be condensed with *cis*–*syn* cyclobutane uracil dimer dicarboxylic acid **5** to afford **2** by using in situ BOP[37] activation of the carboxylic acids and very slow addition of triethylamine to the reaction mixture. To perform measurements in nonpolar solvents such as dioxane, the monoflavin monopentylamide model compound **28** was prepared in a similar fashion through reaction of the *cis*–*syn* cyclobutane dicarboxylic acid **5** with 1 equiv of the flavin ethylamine **9** and subsequent quenching of the reaction mixture with pentylamine.

The ethyl group at the flavin-N(3) center is the only difference between the substitution pattern of our model compounds and of the reaction partners (FAD chromophore and the dimer in damaged DNA) involved in the natural enzymatic repair process. The ethyl group increases the solubility of the model compounds without affecting the redox potential of the riboflavin chromophore.[40] In addition, the X-ray crystal structure of the *E. coli* photolyase reveals no hydrogen bond between the enzyme and the flavin-N(3)–H position, which could alter the redox potential of the isoalloxazine unit in the photolyase flavin-binding pocket.[13]

Investigation of the General Cleavage Properties. For the photolysis experiments in *polar media*, we prepared 10^{-4} M solutions of the model compounds in water, ethylene glycol, or DMF. In these solvents, the flavin chromophore is easily reduced by addition of aqueous sodium dithionite solution. This method affords clear solutions which were subsequently irradiated.

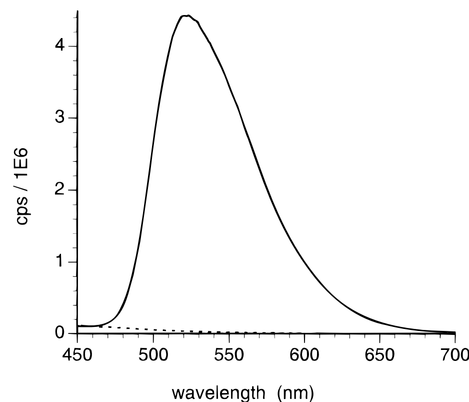


Figure 2. Fluorescence spectra of compound **28** in H_2O (10^{-5} mol/L) (—, **28** oxidized; - - -, **28** reduced) after addition of either sodium dithionite solution or after reduction performed with hydrogen and Pd/barium sulfate catalyst (excitation at 450 nm).

Due to the amphoteric character of the reduced flavin[41,42] and the observation that efficient cleavage of pyrimidine dimers requires a deprotonated flavin chromophore,[19,20] the aqueous reduction solutions were buffered at pH 8.0 with 0.5 M Tris·HCl to ensure the predominant presence of the deprotonated, reduced flavin species ($pK_a = 6.3$). In *apolar media*, reduction of the model compounds was achieved catalytically with hydrogen and Pd on barium sulfate. To ensure that the catalyst causes no interference with the measurements, varying amounts of catalyst were added—within the generally used range—to the reaction mixture. Similar cleavage rates were determined within the experimental error. This catalytic procedure yields clear and optically transparent solutions below 350 nm, which enables exclusive excitation of the reduced flavin chromophores at all required wavelengths. In these measurements, deprotonation of the reduced flavins was accomplished with triethylamine.

As depicted in Figure 2, the fluorescence of the sample is strongly diminished after reduction. This allowed the complete

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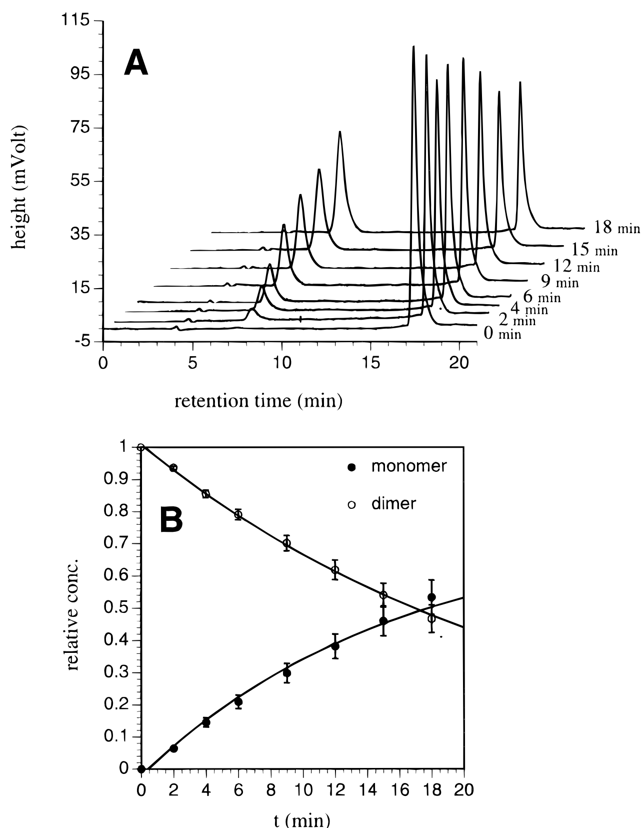

Figure 3. Irradiation experiment performed with **28** in MeOH (3 mL of a 10^{-4} mol/L solution). Reduction was performed by catalytic hydrogenation with Pd/barium sulfate catalyst. Addition of 50 μL of NEt_3 (0.1 M final concentration) ensured complete deprotonation of the reduced flavin chromophores (irradiation at 366 nm). (A) HPLC chromatograms obtained after 0, 2, 4, 6, 9, 12, 15, and 18 min of irradiation (Lichrosphere 100/5 column from Macherey-Nagel). Solvent systems: 55% A and 45% B over 9 min, then switching to 80% A and 20% B for an additional 19 min (A, methanol; B, water) (detection at 450 nm). Retention times: 7.3 min (cleavage product **4**), 17.4 min (model compounds **28**). (B) Plot of relative peak areas vs time and curve fits obtained with an exponential rise to maximum and an exponential decay function.

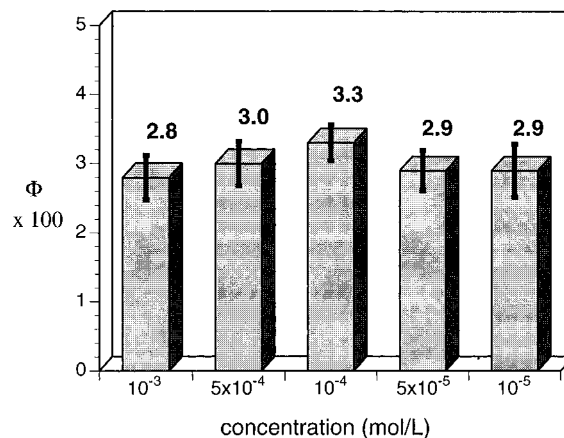

Figure 4. Concentration dependence of the overall splitting quantum yield (ϕ) determined with the model compound **28**. The irradiation experiment was performed in ethylene glycol at 366 nm; reduction was achieved with aqueous dithionite solution (pH 8.0).

reduction of the sample to be monitored during a photolysis experiment.[41-43] As a result of the small, but significant UV spectroscopic differences[43] (vide infra) of the various species (oxidized, reduced, and reduced and deprotonated), the protonation state of the isoalloxazine moiety could be determined precisely before each measurement in all solvents used.[42]

The reduced and deprotonated samples are stable in the dark, in an inert atmosphere for several hours as determined by TLC and by reverse-phase HPLC chromatography of a reoxidized sample. Upon exposure to daylight or to monochromatic light of, for example, 366 or 400 nm, however, the *cis*-*syn* and the *trans*-*syn* model compounds react cleanly to give only one new product. Analysis of the reaction mixture by reverse-phase HPLC and co-injection of the synthesized expected reaction products **3** or **4** confirmed that the light-dependent cleavage reaction yields only **3** and **4**, even after prolonged irradiation.

The splitting rates for the photoinduced cleavage reactions were determined by reverse-phase HPLC analysis of aliquots removed from the irradiated reaction mixture and immediately reoxidized by exposure to oxygen. The series of chromatograms presented in Figure 3A were obtained upon irradiation of model compound **28** in methanol. The time-dependent decrease of the amount of **28** (elution time $\approx$ 17 min) and the increase of the photoproduct **4** (elution time $\approx$ 8 min) are clearly evident. In an effort to quantify the cleavage efficiency, peak areas were integrated and standardized by division through the total peak area. These normalized values were plotted against time as shown in Figure 3B for the measurement presented in Figure 3A. The data points for the amount of starting material **28** were fitted with an exponential decay function. An exponential rise to maximum function was required to fit the data points obtained for the amount of photocleaved product **4**. This proves that the cleavage reaction obeys first-order kinetics, as expected for a purely intramolecular process.

Additional control experiments confirmed the intramolecularity of the cleavage process: (1) Irradiation of mixtures containing reduced methoxyethyl flavin **16** and the *cis*-*syn* cyclobutane uracil dimer dibenzyl esters gave no carboxymethyl uracil benzyl ester, even after prolonged irradiation, and (2) a concentration-dependent investigation of the quantum yield performed with the *cis*-*syn* model compound **28** indicated no change of quantum yields between 10^{-3} and 10^{-5} M concentrations within experimental error (Figure 4). At concentrations of approximately 10^{-4} M, higher quantum yields with a smaller error were obtained because the integration of HPLC peaks was the most precise in this concentration range. Therefore, 10^{-4} M solutions were used for all further measurements.

Two additional control experiments were performed to validate the strict requirement of a reduced flavin sensitizer: (1) Irradiation of the cyclobutane uracil dibenzyl ester alone or in the presence of reducing agents yielded no photoproduct, and (2) irradiation of the model compounds without prior reduction of the flavin chromophore gave no photoconversion even after prolonged irradiation.

To calculate the quantum yields ϕ (ϕ = number of photocleaved model compounds/number of absorbed photons), the initial rates for the cleavage reaction were extracted from the fitted curve. The intensity of the light beam was measured by ferrioxalate actinometry.[44,45] The number of photons absorbed by the model compound was calculated using the absorbance of the irradiated sample at 366 nm, determined prior to the measurement. Quantum yields were calculated on the basis of

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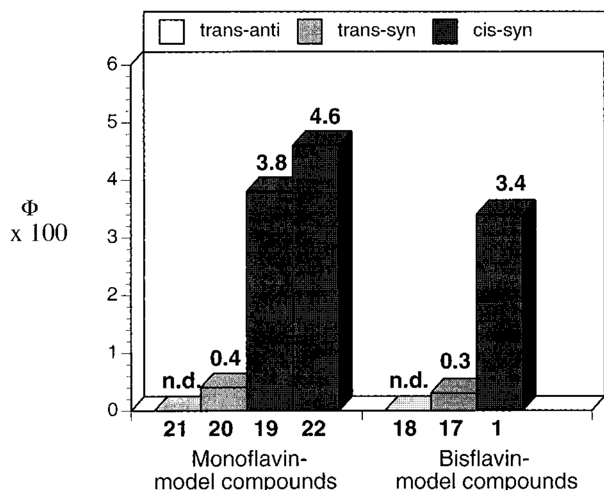

Figure 5. Dependencies of quantum yield on the configuration and constitution of the cyclobutane ring. The *trans*−*anti* (**18** and **21**), *trans*−*syn* (**17** and **20**), and *cis*−*syn* (**1, 19,** and **22**) model compounds were measured in ethylene glycol with irradiation at 366 nm. Reduction was performed with aqueous dithionite solution (pH 8.0).

these results. All photolysis measurement was performed three times, and the averaged data gave an individual error for each measurement of ±8%. Measurements of the parent compounds **1** and **2** in water at pH 8.0 revealed a remarkably high quantum yield of $\phi = 0.064$. This is 2−3 orders of magnitude higher than the quantum yields obtained previously in experiments performed with mixed solutions of riboflavin derivatives and cyclobutane pyrimidine dimers.[17−19] *The present molecules are therefore the first covalently linked flavin*−*pyrimidine dimer model compounds which are able to mimic the enzymatic repair reaction.*

Cleavage of the Cyclobutane Pyrimidine Dimer Depending on the Configuration and Constitution. The photoinduced splitting properties of the three bisflavin model compounds **1**, **17,** and **18**, which contain a *cis*−*syn*, a *trans*−*syn,* or a *trans*−*anti* cyclobutane uracil dimer, were analyzed.[27] All compounds were dissolved in ethylene glycol (sodium dithionite reduction, buffered at pH 8.0) and irradiated at 366 nm (Figure 5). Remarkably different cleavage efficiencies were observed for the three isomers. The fastest cleavage, with a quantum yield of $\phi = 0.034$, was observed for **1**, which contains the *cis*−*syn* cyclobutane uracil dimer. Under identical conditions, a quantum yield of only $\phi = 0.003$ was measured for the corresponding *trans*−*syn* compound **17**, which is thus approximately 10 times more resistant toward the flavin-induced cycloreversion. In agreement with earlier observations,[25] the *trans*−*anti* cyclobutane uracil dimer **18** is unable to undergo the cyclobutane cleavage, even after prolonged irradiation. All three model compounds **1**, **17,** and **18** were also measured in acetonitrile and in ethanol (data not shown) to exclude the possibility of solvent effects. In these solvent systems, similar quantum yield differences were observed for the three cyclobutane uracil dimers. Again, the *cis*−*syn* isomer was found to be approximately 10 times more sensitive toward electron-transfer-induced cycloreversion.

Since flavins are known to build π-stacked aggregates,[38] which might obscure the electron-transfer step,[46] we also investigated the cleavage properties of the four monoflavin model compounds **19**−**22**, in which an intramolecular stacking of two flavin units is impossible. As depicted in Figure 5 increased cleavage rates were again determined for the *cis*−*syn* dimer-containing model compounds **19** and **22**. Due to the absorption of the dithionite at the irradiation wavelength of 366 nm, we checked the results with all model compounds by irradiation at 400 nm, where the dithionite absorption is negligible. Irradiation at 400 nm and also with white light, however, yielded the same relative cleavage rate differences for the *cis*−*syn*, the *trans*−*syn,* and the *trans*−*anti* cyclobutane uracil isomers.

On the basis of our measurements, we conclude that the different flavin-initiated cleavage rates of the three investigated isomers are determined solely by the different cyclobutane constitutions and configurations. The observed increased stability of the *trans*−*syn* isomer most likely also effects the repairability of the natural *cis*−*syn* and *trans*−*syn* DNA lesions by the enzyme DNA photolyase.[2,14] For the enzymatic process, A. Sancar and co-workers determined a very low and probably unspecific binding of the *trans*−*syn* isomer by the *E. coli* photolyases, with a drastically reduced repair quantum yield of $\phi \geq 0.003$ (compare $\phi > 0.6$ for the *cis*−*syn* isomer).[14] Our result suggests that the inherent stability of the *trans*−*syn* dimer might be another factor influencing the low enzymatic repair efficiency.

To gain deeper insight into the possible reason for the increased vulnerability of the *cis*−*syn* dimer toward the flavin-mediated cleavage reaction, we analyzed the crystal structures of the *cis*−*syn* and the *trans*−*syn* isomers for significant differences. C. Pac et al. explained different cleavage rates in oxidative processes as being due to different through-bond interactions of the relevant orbitals in the three isomers.[25] The crucial orbital−orbital overlaps depend critically on the dihedral angle between the interacting orbitals and therefore on the ring pucker. C. Pac suggested that the sterically encumbered *cis*−*syn* dimer causes an increased orbital overlap, which then results in a higher cleavage rate.[25,47]

In the generally accepted view of the reductive splitting process, the donated electron initially occupies the π^* orbital of the C=O bond.[9,48,49] Delocalization of electron density into the antibonding C(5)−C(5′) σ^* orbital weakens this bond and causes the initial bond breakage. For our system, the interaction between the C(4′)=O(4′) and C(4)=O(4) π^* orbitals and the C(5)−C(5′) σ^* orbital is therefore of crucial importance for the cleavage efficiency. Upon electron uptake, the C(5)−C(5′) bond strength could be reduced by different extents in both isomers. This might then cause different cleavage rates. The analysis of the X-ray crystal structures[27] of the *cis*−*syn* and the *trans*−*syn* cyclobutane uracil dimer dibenzyl esters reveals, as expected, only very small differences of the cyclobutane bond lengths and torsion angles. The orientation of the C(4)=O(4) double bond relative to the C(5)−C(5′) bond is, however, significantly different. In both isomers, the descriptive torsion angles O(4)−C(4)−C(5)−C(5′) differ on one side of the dimer by ap-

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(47) Pac, C.; Ohtsuki, T.; Shiota, Y.; Yanagida, S.; Sakurai, H. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 1133−1139. Semiempirical calculations predict unfortunately an almost planar cyclobutane ring and are therefore not able to simulate pucker determined effects. We performed a transition state search using GAMESS(US)[50] on the UHF/PM3 level. For both isomers, the transition states corresponding to the C(5)−C(5′) bond cleavage were found. Frequency analysis verified that the stationary points corresponded to first-order transition states. The activation energies were obtained as the difference between the transition state energies and the energies of the corresponding UHF/PM3-minimized anions. The calculations yielded as expected comparable activation energies of 3.9 kcal/mol for the *cis*−*syn* and 3.6 kcal/mol for the *trans*−*syn* isomer,[49] which underlines the inability of semiempirical calculations to reproduce the observed cleavage rate differences.

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Table 1. Calculated Mayer Bond Orders[51] for the Neutral Dimer and Its Radical Anion, Based on the X-ray Crystal Structure of the Dibenzyl Ester[17,32] with RHF/4-31G-Optimized Hydrogen Atoms as Input Geometry (Program: GAMESS(US))[50]

	neutral dimer		anionic dimer	
isomer	C(5)−C(5′) bond	C(6)−C(6′) bond	C(5)−C(5′) bond	C(6)−C(6′) bond
cis−syn				
UHF/STO-3G	0.958	0.945	0.884	0.940
UHF/6-31G*	0.947	0.938	0.778	0.936
UHF/DZV	0.850	0.852	0.692	0.857
trans−syn				
UHF/STO-3G	0.963	0.931	0.906	0.924
UHF/6-31G*	0.964	0.914	0.826	0.924
UHF/DZV	0.890	0.870	0.748	0.884

proximately 20°! To investigate how this difference might influence the cleavage properties, ab initio calculations were performed using the program GAMESS(US).[50] The X-ray crystal structures were used as geometries for modeling both the neutral molecules and their radical anions. The calculated Mayer bond orders are listed in Table 1.[51] Electron transfer to both isomers results in the expected reduction of the C(5)−C(5′) bond orders. The decreases in bond order were calculated to be approximately 19% for the *cis−syn* isomer and, on average, only 15% for the *trans−syn* dimer. The calculations therefore show the expected trend. The bond weakening is more pronounced in the *cis−syn* dimer radical anion, and this isomer is cleaved 10 times faster than the *trans−syn* dimer. More detailed calculations are now required to fully understand and to quantify the increased lability of the *cis−syn* isomer. Nevertheless, the rudimentary bond order calculations suggest an attractive preliminar explanation for the observed rate differences.[25]

Medium-Dependent Measurements of the Cleavage Reaction.[52] To investigate how the polar flavin-binding pocket of the *E. coli* photolyase might influence the cleavage reaction, medium-dependent measurements were performed with the monoflavin−monopentylamide model compound **28**. To validate the results, further measurements were carried out with the bisflavin model compound **2**.

Initially, the cleavage efficiency was investigated in pure water and the polarity of the medium was reduced by addition of ethylene glycol or ethanol (data not shown): these solvents possess similar characteristics but have different dielectric constants. We also measured the cleavage efficiency in pure ethylene glycol and in DMF (Figure 6). The required aqueous solutions were buffered at pH 8.0. UV/vis measurements performed prior to the irradiation experiments ensured complete reduction and deprotonation of the flavin units. The highest quantum yield (ϕ = 0.062) in the cleavage reaction of **28** was obtained in pure water. Upon addition of ethylene glycol, a gradual decrease of the quantum yield was observed until ϕ = 0.033 was reached in pure ethylene glycol. The systematic reduction of the medium polarity results in a significant decrease of the quantum yield for the cleavage reaction. Decreasing the solvent polarity extends the half-life of the model compound **28** from 10 min in water to 20 min in ethylene glycol. A second series of measurements performed with the bisflavin amide model compound **2** (Figure 6) confirmed the small solvent dependence and increased cleavage rates in the most polar solvent mixture H_2O/ethylene glycol (2:1).

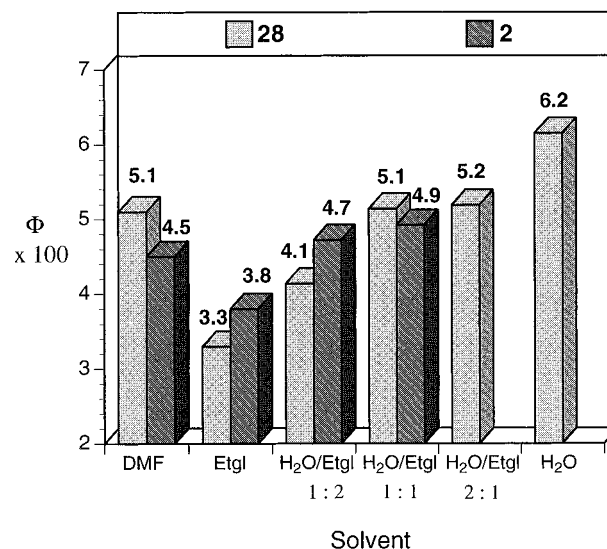

Figure 6. Measurement of the dependency of the quantum yield on the polarity of the medium with the model compounds **2** and **28**. Irradiation was performed at 366 nm; reduction was achieved by the addition of aqueous dithionite solution buffered at pH 8.0. The measurements were performed in water (ϵ = 78.3), in water/ethylene glycol (Etgl) mixtures, in pure ethylene glycol (ϵ = 37.7), and in DMF (ϵ = 36.7).[52]

Table 2. Solvent Dependence of Quantum Yields for Compound **28**

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.[52] [b] Quantum yields of model compound **28**. [c] No quantum yield detectable even after prolonged irradiation.

In addition, the cleavage efficiency was measured in various organic solvents. The model compound **28** was catalytically reduced, and triethylamine (50 μL, final concentration 0.1 M) was added to ensure complete deprotonation of the flavin chromophore (determined by UV/vis spectroscopy). The quantum yields calculated for the cleavage reaction in various organic solvents are given in Table 2. In these measurements, compound **28** features the highest quantum yields in the most polar solvents. A gradual decrease of the cleavage efficiency was observed in organic solvents as a function of decreasing polarity.

The irradiation experiments performed in organic media support the results obtained in water/ethylene glycol mixtures and suggest that the appearance of highly charged intermediates during the cleavage reaction is unlikely.[53] We observe only a 4-fold decrease of the quantum yield over the entire polarity range investigated. The best cleavage efficiencies were observed in polar media. The result of the polarity-dependent measurement is in perfect agreement with the X-ray crystal structure of the *E. coli* photolyase, which shows a riboflavin

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(53) Another explanation for the observed solvent effects is that the conformation of the cyclobutane uridine dimer is altered in different media. Although this cannot be ruled out, the descriptive torsion O(4)−C(4)−C(5)−C(5′), which is responsible for the orbital overlap, must change by several degrees in order to influence the cleavage rate by a factor of 4. We believe currently that such a torsion angle change, within a rigid cyclobutane pyrimidine dimer framework, is unlikely.

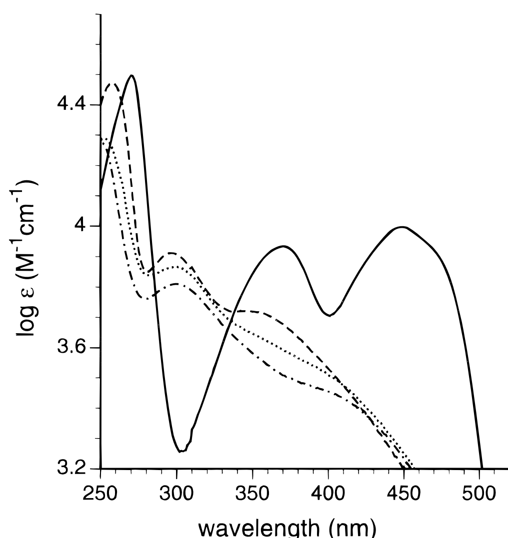


Figure 7. UV spectra of the model compound **28** in H_2O (10^{-5} mol/L): reduction was performed by catalytic hydrogenation over Pd/barium sulfate catalyst [—, oxidized **28** (pH 3−10); - • -, reduced **28** (pH 3−5); •••, reduced **28** (pH 6); - - -, reduced **28** (pH 7−10)]. Citrate buffers (0.5 M) were used for pHs 3, 4, 5, and 6. Tris·HCl buffers (0.5 M) were employed for pHs 7, 8, 9, and 10.

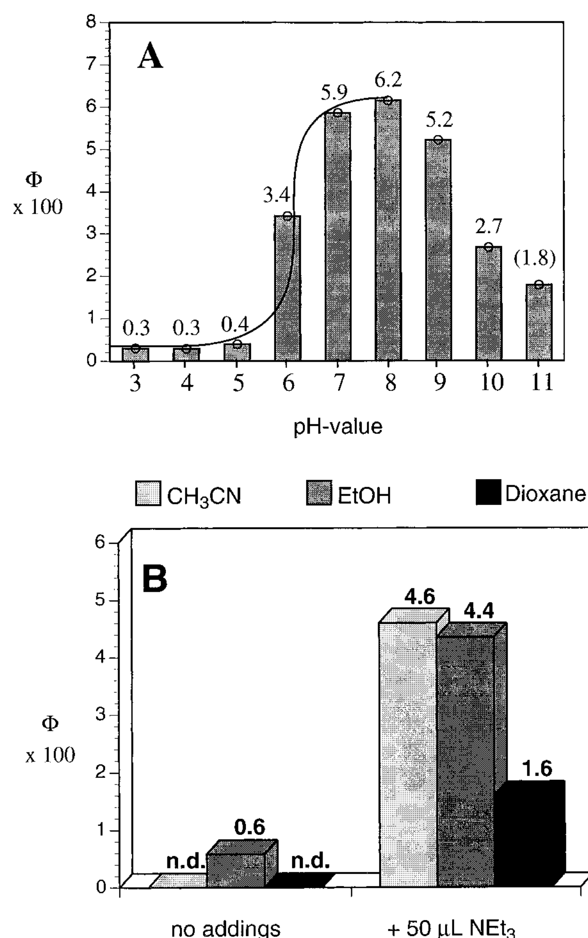


Figure 8. (A) pH dependence of quantum yields measured with **28** in H_2O at different pH values (0.5 M citrate buffers for pHs 3, 4, 5, and 6; 0.5 M Tris·HCl buffers for pHs 7, 8, and 9; 0.05 M phosphate buffers for pHs 10 and 11). The value at pH 11 possesses a greater error due to beginning of decomposition of the model compound. (B) Measurement of the quantum yield of **28** in acetonitrile, ethanol, and dioxane. Right side: in the presence of triethylamine. Left side: in the absence of triethylamine.

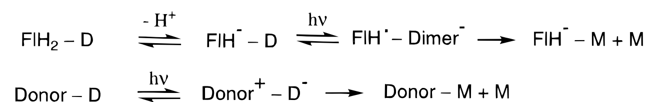
cofactor tightly bound in an unusually polar binding pocket by an array of hydrogen bonds.[13] A salt bridge is oriented parallel to the isoalloxazine chromophore within van der Waals contact. Several water molecules are in close proximity to the isoalloxazin ring system, and both heterocycles of the FAD cofactor are partly solvent-accessible through an opening in the surface of the enzyme. The fact that the amino acids involved in the flavin-binding pocket are highly conserved in all eight known microbial photolyases suggests that the polar environment of the cofactor is of general importance for the enzymatic repair reaction.[13] On the basis of the solvent-dependent studies, we presume that this polar environment might be in part responsible for the high catalytic efficiency observed for these enzymes.

We believe that the solvent effect measured with our model compounds is related to the ability of the system to deprotonate the reduced riboflavin. The reduced riboflavin has a low pK_a value ($pK_a = 6.3$),[21] allowing it to be readily deprotonated under physiological conditions, thus generating a negatively charged electron donor (FlH^-). pH-dependent measurements were carried out to further investigate the dependence of the cleavage reaction in our model compounds on the degree of protonation of the reduced flavin.

pH-Dependent Measurements of the Cleavage Reaction. The cleavage reaction was investigated in water at various pH values and in different organic solvents such as acetonitrile, ethanol, or dioxane in the presence of acetic acid or triethylamine. The degree of protonation of the reduced flavin was closely monitored by UV spectroscopy. Figure 7 depicts the UV spectra obtained from 10^{-5} M solutions of oxidized **28** and the hydrogenated model compound **28** below pH 5 (neutral species), at pH 6, and above pH 7 (deprotonated species). The measured spectra are in agreement with the pK_a value of 6.3[21] determined for the reduced riboflavin and with literature spectra obtained for similar flavin species.[43] In organic media, we observed the reduced and protonated flavin species directly after catalytic reduction and also in the presence of acetic acid. Addition of triethylamine yielded the deprotonated form, as determined by UV spectroscopy. These solutions (10^{-4} M) were then irradiated at 366 nm. The calculated quantum yields are depicted in Figure 8A,B. In organic solvents, efficient splitting of the cyclobutane ring is strictly limited to solutions containing triethylamine. In solutions without base, the measured photocleavage is very low. Addition of acetic acid during an irradiation experiment caused the immediate termination of the cleavage reaction. Thus the reduced, neutral riboflavin species is unable to perform photocleavage. This observation is in agreement with earlier measurements performed by S. D. Rose and co-worker[20] and by M. S. Jorn[19] with flavin and cyclobutane pyrimidine dimer solutions. Supporting results were obtained by measurements performed with buffered aqueous solution. Here, we observed negligible splitting below pH 5 and maximal splitting efficiencies above pH 7. The half-maximal quantum yield was observed around pH 6. In contrast to earlier studies,[20] the model compounds are repaired with close to maximum efficiency at pH 7. These results show that maximal cleavage efficiency is reached under physiological conditions. At pH 9, the cleavage efficiency decreases again, possibly due to the deprotonation of the cyclobutane uracil dimer unit (pK_a = 10.7).[19] Above pH 11, decomposition of the model compounds was observed, thus preventing further measurements.

Conclusions of the Solvent and pH-Dependent Measurements. Earlier medium-dependent measurements with model compounds which possess an arylamine- or indol-type electron donor, instead of a reduced and deprotonated flavin cofactor, showed increased cleavage efficiencies in nonpolar media such as hexane/dioxane (95:5).[28,29] These previously investigated [Donor−Dimer] systems yield a zwitterionic intermediate

Scheme 3. Schematic Representation of the Cleavage Process in a Donor Cyclobutane Pyrimidine Dimer System (Donor−D) and in a Flavin Cyclobutane Pyrimidine Dimer System (FlH_2−D) (D, Dimer; M, Monomer)



[Donor$^-$−Dimer$^+$] after photoinduced electron transfer to the dimer unit. It is currently believed that this intermediate possesses a high driving force for an unproductive reversal of the electron-transfer process, which then regenerates the [Donor−Dimer] state.[28,29] On the basis of the observation that the cleavage in these systems proceeds most efficiently in nonpolar media, it was postulated that the competing back electron transfer might be slowed in the Marcus inverted region.[29]

In contrast, a photoinduced electron transfer from a deprotonated flavin to the pyrimidine dimer [FlH$^-$−Dimer] results in a simple shift of the negative charge from the flavin unit to the dimer [FlH$^\bullet$−Dimer$^-$] as depicted in Scheme 3. The occurrence of a [FlH$^\bullet$−Dimer$^-$] intermediate, which is not significantly more or less polar than the initial state, is strongly supported by the minimal medium dependence observed for the cleavage reaction (factor four). Such an intermediate was previously suggested by T. Okamura et al. based on picosecond laser photolysis studies.[54] The lifetime of the [FlH$^\bullet$−Dimer$^-$] state is of crucial importance for the efficient photoinduced cycloreversion of cyclobutane pyrimidine dimers by reduced and deprotonated flavins. The [FlH$^\bullet$−Dimer$^-$] intermediate can either split to yield [FlH$^-$−M + M] or can collapse by a back electron transfer to give [FlH$^-$−Dimer]. Due to the nonzwitterionic character of this intermediate, a much smaller driving force is expected for the unproductive charge-shift reaction. These considerations and the medium-dependent measurements seem to exclude the "Marcus inverted region" argument as an explanation for the high repair efficiency in flavin/pyrimidine dimer systems. It is possible, however, that the polar environment and the known stability of the FADH$^\bullet$ are the crucial factors influencing the stability of the [FADH$^\bullet$−Dimer$^-$] intermediate. In the enzymatic case, the FADH$^\bullet$ is known to be exceptionally well stabilized if bound in the enzymatic binding pocket,[9] and most purified photolyases contain the riboflavin cofactor in its neutral blue radical form. This indicates that the FADH$^\bullet$ stability might also be important for the enzymatic repair efficiency.

Summary and Outlook. The model compounds presented herein mimic the photoinduced, reduced flavin-dependent cleavage of cyclobutane pyrimidine dimers ($\phi = 0.06$). This process is exploited by nature to remove the harmful cyclobutane pyrimidine dimer lesion present in UV-B-irradiated DNA. The investigation of the cleavage reaction with differently configured cyclobutane uracil dimers revealed an enhanced cleavage efficiency for the *cis*−*syn* dimer, which is the major substrate for DNA photolyases. The *trans*−*syn* cyclobutane uracil dimer demonstrates an increased resistance toward flavin-induced reductive cycloreversion. This resistance might be one factor influencing the low *trans*−*syn* repair activity observed for the *E. coli* DNA photolyase.[14]

Investigation of the solvent-dependent cleavage process revealed that reaction rates increase in polar media. These studies show that the polar flavin environment observed in the X-ray structure of the *E. coli* enzyme[13] may be required to maximize the repair efficiency. The dependence of the cleavage reaction on the protonation state of the reduced flavin is in agreement with earlier observations[19,20] and underlines the strict necessity to deprotonate the reduced flavin chromophore. The model compounds reveal maximal repair efficiency under physiological conditions (pH > 7), which is in agreement with the pK_a value of 6.3[21] for the reduced riboflavin. Above pH 9, the quantum yield decreases, due to deprotonation of the dimer unit.

The spectral region between 190 and 360 nm is currently not accessible in measurements performed with the enzyme because the enzyme requires sulfite containing buffers for the reduction of the enzyme-bound flavin. Reduction of our model compounds by catalytic hydrogenation enables the preparation of active model compound solutions with an optical transparancy from 190 to 360 nm. This possibility allows, for the first time, the investigation of transient intermediates which possess absorption bands in this spectral region and the measurement of their lifetimes by picosecond laser photolysis spectroscopy. The results of these measurements will be reported in due course.

Experimental Section

General Methods. All materials were obtained from commercial suppliers and were used without further purification. Solvents of technical quality were distilled prior to use. The aqueous buffers were prepared using deionized distilled water. For reactions under an inert gas atmosphere, nitrogen of standard quality was used. For analytical thin layer chromatography, precoated silica gel plates (Merck 60-F254) were used. Staining of amino compounds was performed with ninhydrin. Flash chromatography was performed using Silica gel (Merck, 0,040−0.063 mm) and Silica gel-*H* (Fluka, 0.005−0.040 mm). Melting points are uncorrected and were determined on a Büchi Smp 20. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR using KBr pellets or $CHCl_3$ solutions. UV spectra were recorded on a Varian Cary 5 UV/vis spectrophotometer in 1 cm quartz cuvettes. Fluorescence spectra were measured on a Spex 1680 0.22 m double spectrometer with a bandwidth of 3.4 nm in 1 cm quartz cuvettes. NMR spectra were recorded on a Varian Gemini 200 (200 MHz (^{1}H), 50 MHz (^{13}C)), Varian Gemini 300 (300 MHz (^{1}H), 75 MHz (^{13}C)), and Bruker AM-500 (500 MHz (^{1}H), 125 MHz (^{13}C)). The chemical shift (δ) is reported in parts per million downfield from tetramethylsilane (TMS, $\delta = 0$ ppm). Altermatively, the resonance of residual solvent protons were used as the reference. EI-mass spectra and FAB-mass spectra were measured by the staff of the mass spectrometry facilities of the ETH Zurich on a Hitachi-Perkin-Elmer VG TRIBRID with 70 eV ionization energy (EI) and on a ZAB-2 SEQ with 3-nitrobenzyl alcohol as the matrix (FAB). Elemental analysis were performed by the microanalysis laboratory of the ETH Zurich. HPLC chromatograms were obtained with a Knauer HPLC instrument (HPLC pumps 64, Knauer variable-wavelength UV detector, Knauer degasser) using HPLC grade solvents.

***N*-(2′-Methoxyethyl)-4,5-dimethyl-2-nitroaniline (13).** 1-Bromo-2-methoxyethane[37] (**12**) and 4,5-dimethyl-2-nitro-*N*-(trifluoroacetyl)-aniline[36] (**11**) were prepared according to the literature. A solution containing **11** (8.00 g, 30.5 mmol) and K_2CO_3 (16.0 g, 116 mmol) 100 mL in DMF (100 mL) was heated to 100 °C. At this temperature, 1-bromo-2-methoxyethane (**12**) (12.0 g, 86.3 mmol) was carefully added. The solution was kept at reflux temperature for additional 90 min. Then the solvent was removed in vacuo. Water was added to the residual material, and the slurry was extracted with 250 mL of $CHCl_3$ three times. The combined organic layers were dried with $MgSO_4$, filtered, and concentrated in vacuo. Isolation of the product by silica gel flash column chromatography (6 × 12 cm, toluene → toluene/EtOAc (10:1)) afforded **13** (6.12 g, 89%) as an orange solid: mp 33−35 °C; IR ($CHCl_3$) 3378 (w), 3011 (w), 2922 (w), 1632 (s), 1574 (s), 1507 (s),1409 (m), 1336 (m), 1241 (s), 1156 (m), 1121 (m), 1022 (w), 1006 (w), 850 (w) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.18 (s, 3 H), 2.27 (s, 3 H), 3.43 (s, 3 H), 3.49 (t, $J = 5.4$ Hz, 2 H),

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3.67 (t, $J = 5.4$ Hz, 2 H), 6.64 (s, 1 H), 7.93 (s, 1 H), 8.10 (m, 1 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 18.73, 20.91, 43.01, 59.31, 70.84, 114.38, 114.43, 124.86, 126.88, 144.40, 147.53; EI-MS (70 eV) *m/z* 224 (20, M − H$^+$), 179 (100, [M − NO_2]$^+$), 106 (15), 28 (6).

10-(2′-Methoxyethyl)-7,8-dimethyl-10*H*-benzo[*g*]pteridine-2,4-dione (15). A suspension of Pd/C catalyst (50 mg) in AcOH (2 mL) was slowly added to a solution of **13** (3.00 g, 13.3 mmol) in 50 mL of AcOH. The mixture was sparged with hydrogen and stirred for 15 h at room temperature. The reaction mixture, containing the diamine **14**, was filtered through a Celite pad. Then alloxane monohydrate (7.00 g, 43.7 mmol) and boric acid (15.0 g, 243 mmol) were added. The yellow slurry was stirred for 6 h at room temperature. Next, 500 mL of water were added and the reaction mixture was extracted three times with 250 mL of $CHCl_3$ each. The organic layers were combined, washed with saturated aqueous Na_2CO_3 solution, dried with $MgSO_4$, and concentrated in vacuo. Recrystallization of the residual material from AcOH/H_2O gave **15** (1.79 g, 46%) as a gold brown powder: mp 279−281 °C (dec); IR ($CHCl_3$) 3378 (w), 3010 (w), 1717 (m), 1677-(m), 1600 (w), 1581 (m), 1547 (s), 1344 (w), 1261 (w), 1117 (w) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.45 (s, 3 H), 2.55 (s, 3 H), 3.30 (s, 3 H), 3.92 (t, $J = 5.2$ Hz, 2 H), 4.91 (t, $J = 5.2$ Hz, 2 H), 7.69 (s, 1 H), 8.04 (s, 1 H), 8.62 (br.s, 1 H); ^{13}C NMR (50 MHz, $(CD_3)_2SO$) δ 18.37, 20.28, 43.50, 58.06, 67.83, 116.29, 130.38, 130.98, 133.25, 135.35, 136.70, 145.86, 149.83, 155.12, 159.48; EI-MS (70 eV) *m/z* 300 (3, M$^+$), 242 (100, [M − C_3H_6O]$^+$), 171 (80), 156 (28), 44 (34). Anal. Calcd for $C_{15}H_{16}N_4O_3 \cdot {}^1/_2H_2O$ (309.32): C, 58.25; H, 5.50; N, 18.12. Found: C, 58.77; H, 5.42; N, 18.03.

3-Ethyl-10-(2′-methoxyethyl)-7,8-dimethyl-10*H*-benzo[*g*]pteridine-2,4-dione (16). A solution of iodoethane (2.50 g, 16.0 mmol) was added dropwise to a mixture of **15** (1.09 g, 3.63 mmol) and K_2CO_3 (3.00 g, 21.7 mmol) 50 mL in DMF. The reaction slurry was stirred overnight at room temperature, then diluted with 500 mL of water and extracted with 200 mL of $CHCl_3$ each. The combined organic extracts were dried with $MgSO_4$, filtrated, and concentrated in vacuo. The residual material was subjected to flash chromatography on silica gel (3 × 15 cm, acetone/CH_2Cl_2 (1:1)) to give **16** as an orange oil. Recrystallization from MeOH/H_2O afforded **16** (0.990 g, 83%) as orange platelets: mp 204−205 °C; IR ($CHCl_3$) 3000 (w), 1706 (w), 1651 (m), 1583 (m), 1548 (s), 1439 (w), 1339 (w), 1117 (w) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) $\delta = 1.31$ (t, $J = 7.1$ Hz, 3 H), 2.43 (s, 3 H), 2.53 (s, 3 H), 3.29 (s, 3 H), 3.91 (t, $J = 5.3$ Hz, 2 H), 4.17 (q, $J = 7.1$ Hz, 2 H), 4.88 (t, $J = 5.3$ Hz, 2 H), 7.65 (s, 1 H), 8.02 (s, 1 H); ^{13}C NMR (50 MHz, $CDCl_3$) $\delta = 12.78$, 19.18, 21.25, 36.87, 44.94, 58.93, 69.22, 116.28, 131.85, 131.92, 134.63, 135.39, 136.20, 147.03, 148.40, 155.17, 159.41; EI-MS (70 eV) *m/z* 328 (4, M$^+$), 270 (100), 242 (23), 199 (32), 171 (22), 44 (16). Anal. Calcd for $C_{17}H_{20}N_4O_3$ (328.37): C, 62.18; H, 6.14; N, 17.06. Found: C, 62.23; H, 6.13; N, 17.05.

3-Ethyl-10-(2′-hydroxyethyl)-7,8-dimethyl-10*H*-benzo[*g*]pteridine-2,4-dione (8). A solution of **16** (0.980 g, 2.71 mmol) in CH_2Cl_2 (50 mL) was cooled to 0 °C. Then BBr_3 (3.5 mL, 36.3 mmol) dissolved in 10 mL of CH_2Cl_2 was slowly added with a syringe under nitrogen. The solution was stirred at 0 °C for an additional 4.5 h. A mixture of H_2O (2.5 mL) and acetone (5 mL) was added dropwise and very carefully to hydrolyze unreacted BBr_3. The mixture was stirred for another 15 min, then 300 mL of acetone and 200 mL of H_2O were added. The organic solvents were removed in vacuo. The residual aqueous product solution was allowed to stay at 0 °C for 24 h. The precipitate **8** was removed by filtration and recrystallized from acetone/H_2O. **8** (0.690 g, 81%) was obtained in form of yellow needles: mp: 205−208 °C (dec); IR ($CHCl_3$) 3689 (w), 3011 (s), 1706 (w), 1650 (m), 1583 (m), 1548 (s), 1461 (w), 1439 (w), 1339 (w), 1133 (m), 928 (w), 622 (m) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.27 (t, $J = 7.1$ Hz, 3 H), 2.44 (s, 3 H), 2.54 (s, 3 H), 3.00 (t, $J = 5.5$ Hz, 1 H), 4.09 (q, $J = 7.1$ Hz, 2 H), 4.21 (t, $J = 5.5$ Hz, 2 H), 4.92 (t, $J = 5.5$ Hz, 2 H), 7.64 (s, 1 H), 8.02 (s, 1 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 13.20, 19.67, 21.76, 37.37, 47.39, 60.18, 116.28, 132.05, 132.78, 135.43, 137.08, 137.30, 148.27, 149.45, 155.72, 159.84; EI-MS (70 eV) *m/z* 314 (1, M$^+$), 298 (13), 270 (43), 199 (40), 171 (25), 77 (27), 44 (100). Anal. Calcd for $C_{16}H_{18}N_4O_3 \cdot 1H_2O$ (332.34): C, 57.77; H, 6.02; N, 16.85. Found: C, 57.80; H, 5.62; N, 16.63.

4,5-Dimethyl-2-nitro-*N*-(2′-phthalimidoethyl)aniline (24). 4,5-Dimethyl-2-nitroaniline (**10**, 3.00 g, 18.1 mmol) and an excess of *N*-(2-bromoethyl)phthalimide (**23**, 8.00 g, 31.5 mmol) were mixed and pulverized. This solid material was heated in a culture tube at 175 °C for 5 h. The melted solids were allowed to cool to room temperature. The obtained solid material was treated with 200 mL of boiling EtOH. The obtained slurry was heated to reflux for 10 min and subsequently sonicated at room temperature for an additional 30 min to dissolve all product. The slurry was allowed to stand at 4 °C overnight. The brown precipitate was filtered off and recrystallized from EtOH twice. **24** (1.44 g, 23.5%) was obtained as brownish needles: mp 213-215 °C; IR ($CHCl_3$) 3378 (w), 1772 (w), 1717 (s), 1633 (w), 1572 (m), 1506 (m), 1394 (m), 1339 (w), 1122 (m), 1106 (m) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) $\delta = 2.16$ (s, 3 H), 2.28 (s, 3 H), 3.63 (q, $J = 6.3$ Hz, 2 H), 4.01 (t, $J = 6.3$ Hz, 2 H), 6.80 (s, 1 H), 7.74 (m, 2 H), 7.87 (m, 2 H), 7.92 (s, 1 H), 8.10 (q, $J = 6.3$ Hz, 1 H); ^{13}C NMR (50 MHz, $CDCl_3$) $\delta = 18.72$, 20.91, 36.82, 41.55, 114.36, 123.83 (2C), 125.37, 126.94, 130.75 (2C), 132.26, 134.55 (2C), 143.86, 147.73, 168.65 (2C); EI-MS (70 eV) *m/z* 339 (13, M$^+$), 304 (8), 179 (100), 160 (28), 147 (11), 133 (10), 104 (16), 77 (21). Anal. Calcd for $C_{18}H_{17}N_3O_4 \cdot 1H_2O$ (357.36): C, 60.50; H, 5.36; N, 11.76. Found: C, 60.47; H, 4.91; N, 11.75.

7,8-Dimethyl-10-(2′-phthalimidoethyl)-10*H*-benzo[*g*]pteridine-2,4-dione (26). **24** (200 mg, 0.589 mmol) was dissolved in 20 mL of acetic acid (20 mL) at 50 °C. At this temperature, a suspension of Pd/C catalyst (15 mg) in AcOH (1 mL) was added to the reaction solution. The mixture was kept under hydrogen at 50 °C for 30 h. The colorless solution was subsequently allowed to cool to room temperature and filtered through a Celite pad. Alloxane monohydrate (400 mg, 2.50 mmol) and boric acid (800 mg, 12.96 mmol) were added, and the slurry was stirred at room temperature for an additional 16 h. The suspension was diluted with 500 mL of water and extracted with 200 mL of $CHCl_3$. The combined organic layers were dried with $MgSO_4$ and concentrated in vacuo. The resulting yellow oil was solidified through addition of 200 mL of diethyl ether. The solid product was filtered off to yield **26** (115 mg, 47%) as a yellow powder: mp 298−300 °C; IR ($CHCl_3$) 3378 (w), 1772 (w), 1717 (s), 1633 (w), 1583 (m), 1550 (s), 1394 (m), 1244 (m), 1167 (w), 989 (m), 894 (m) cm^{-1}; ^{1}H NMR (300 MHz, $(CD_3)_2SO$) δ 2.34 (s, 3 H), 2.40 (s, 3 H), 4.03 (t, $J = 6.0$ Hz, 2 H); 4.91 (t, $J = 6.0$ Hz, 2 H), 7.79 (m, 4 H), 7.82 (s, 1 H), 7.88 (s, 1 H), 11.26 (s, 1 H); MS (FAB$^+$) 416 (100, [M + H]$^+$), 391 (4), 371 (7), 242 (4), 174 (5), 107 (12). Anal. Calcd for $C_{22}H_{17}N_5O_3 \cdot 2H_2O$ (451.44): C, 58.53; H, 4.69; N, 15.51. Found: C, 58.70; H, 4.39; N, 15.59.

3-Ethyl-7,8-dimethyl-10-(2′-phthalimidoethyl)-10*H*-benzo[*g*]pteridine-2,4-dione (27). Iodoethane (0.75 mL, 9.28 mmol) was added to a suspension of **26** (240 mg, 0.578 mmol) and dry K_2CO_3 (800 mg, 5.79 mmol) in 20 mL of DMF. The reaction slurry was stirred for 24 h at room temperature. The mixture was diluted with 100 mL of water and extracted with 200 mL of $CHCl_3$ three times. The combined organic layers were dried with $MgSO_4$, filtrated, and concentrated in vacuo. The crude residue was subjected to flash chromatography with silica-*H* adsorbent (3 × 20 cm, $CHCl_3$/MeOH (12:1)). **27** (220 mg, 86%) was obtained as a yellow powder: mp 254−256 °C; IR ($CHCl_3$) 1772 (w), 1716 (s), 1656 (m), 1589 (m), 1549 (s), 1439 (w), 1394 (m), 1333 (w) cm^{-1}; ^{1}H NMR (300 MHz, $(CD_3)_2SO$) δ 0.95 (t, $J = 6.9$ Hz, 3 H), 2.33 (s, 3 H), 2.39 (s, 3 H), 3.75 (q, $J = 6.9$ Hz, 2 H), 4.00 (t, $J = 5.6$ Hz, 2 H), 4.93 (t, $J = 5.6$ Hz, 2 H), 7.75 (m, 4 H), 7.84 (s, 1 H), 7.91 (s, 1 H); ^{13}C NMR (75 MHz, $(CD_3)_2SO$) $\delta = 12.70$, 18.60, 20.54, 34.66, 35.71, 41.87, 115.86, 123.13 (2C), 130.78, 131.38, 131.47 (2C), 134.24, 134.49 (2C), 136.08 (2C), 147.03, 149.28, 154.11, 158.96, 167.98 (2C); MS (FAB$^+$) 444 (100, [M + H]$^+$). Anal. Calcd for $C_{24}H_{21}N_5O_4 \cdot 1H_2O$ (461.48): C, 62.47; H, 5.02; N, 15.18. Found: C, 62.59; H, 4.69; N, 15.12.

3-Ethyl-7,8-dimethyl-10-(2′-aminoethyl)-10*H*-benzo[*g*]pteridine-2,4-dione (9). A suspension of **27** (455 mg, 1.03 mmol) in 30 mL of concentrated HCl was stirred at reflux temperature for 4 h. The reaction mixture was concentrated in vacuo. The residue material was triturated with 20 mL of MeOH and filtrated. The solid material was washed with acetone twice to yield **9** (330 mg, 92%) as a yellow powder: mp 286−288 °C; IR ($CHCl_3$) 3444 (m), 1713 (s), 1615 (s), 1580 (s), 1542 (s), 1458 (m), 1343 (m), 1250 (s), 1193 (m), 1022 (w), 929 (w), 878 (w), 806 (w), 773 (w), 443 (w) cm^{-1}; ^{1}H NMR (300 MHz, $(CD_3)_2SO$) δ 1.16 (t, $J = 6.9$ Hz, 3 H), 2.42 (s, 3 H), 2.50 (s, 3 H), 3.20 (m, 2 H),

3.94 (q, $J = 6.9$ Hz, 2 H), 4.91 (t, $J = 6.0$ Hz, 2 H), 7.98 (s, 1 H), 8.05 (s, 1 H), 8.17 (m, 3 H); ^{13}C NMR (75 MHz, $(CD_3)_2SO$) δ 12.82, 18.63, 20.41, 35.93, 35.99, 41.11, 116.05, 130.68, 131.28, 134.30, 136.20, 136.46, 147.31, 149.63, 154.68, 159.25; MS (FAB$^+$) 314 (100, [M-Cl]$^+$), 271 (15), 232 (7).

General Procedure for the Preparation of the 1-(Carboxymethyl)uracil Cyclobutane Dimer Bisflavin Esters (1, 17, and 18). The synthesis of the 1-(carboxymethyl)uracil cyclobutane dimer starting materials **5**−**7** was achieved as decribed by us recently.[17] BOP (150 mg, 0.323 mmol) was added to a solution of the corresponding *cis*−*syn*, *trans*−*syn*, or *trans*−*anti* 1-(carboxymethyl)uracil cyclobutane dimers (**5**−**7**, 50 mg, 0.147 mmol) in 1.5 mL of DMF. This solution was stirred at room temperature for 10 min. Then a solution of **8** (100 mg, 0.318 mmol) in 3 mL of DMF was added to the activated dimer solution. Ten drops of triethylamine were added to start the reaction, which was stirred at room temperature for 24 h. The reaction mixture was diluted with 100 mL of water and extracted with 200 mL of $CHCl_3$ three times. The combined organic layers were dried with $MgSO_4$, filtrated, and concentrated in vacuo. The remaining orange oil was solidified through trituration with diethyl ether. The residual material was subjected to flash chromatography with silica-*H* as adsorbent (3 × 15 cm, $CHCl_3$/MeOH (10:1)). The yields for the three model compounds **1**, **17**, and **18** were 67 mg (49%) for **1**, 118 mg (84%) for **17,** and 36 mg (27%) for **18**. All compounds were obtained as a yellow powder.

1-(Carboxymethyl)uracil (*cis*−*syn*)-Cyclobutane Dimer Bisflavin Ester (1): HRMS (FAB$^+$) m/z calcd for $C_{44}H_{45}N_{12}O_{12}$ ([M + H]$^+$) 933.3280, found 933.3320. For all other analytical data see ref 27.

1-(Carboxymethyl)uracil (*trans*−*syn*)-Cyclobutane Dimer Bisflavin Ester (17): HRMS (FAB$^+$) m/z calcd for $C_{44}H_{45}N_{12}O_{12}$ ([M + H]$^+$) 933.3280, found 933.3441. For all other analytical data see ref 27.

1-(Carboxymethyl)uracil (*trans*−*anti*)-Cyclobutane Dimer Bisflavin Ester (18): HRMS (FAB$^+$) m/z calcd for $C_{44}H_{45}N_{12}O_{12}$ ([M + H]$^+$) 933.3280, found 933.3412. For all other analytical data see ref 27.

General Procedure for the Preparation of 1-(Carboxymethyl)uracil Cyclobutane Dimer Monobenzyl Ester Monoflavin Ester (19−**21).** A solution of the corresponding (*cis*−*syn*, *trans*−*syn,* or *trans*−*anti*)-1-(carboxymethyl)uracil cyclobutane dimers (**5**−**7**, 100 mg, 0.294 mmol) and an excess of BOP (300 mg, 0.646 mmol) were dissolved in 4 mL of DMF and stirred at room temperature for 10 min. After the addition of a solution of **8** (46 mg, 0.147 mmol) in 2 mL DMF (2 mL) and of 10 drops of triethylamine, the reaction was stirred for 5 h at room temperature. Then benzyl alcohol (40 mg, 0.38 mmol) was added to the reaction mixture, which was stirred for an additional 20 h at room temperature. The reaction mixture was diluted with 100 mL of water and extracted with 200 mL of $CHCl_3$ three times. The combined organic layers were dried with $MgSO_4$, filtrated, and concentrated in vacuo. The remaining orange oil was solidified through trituration with diethyl ether. The solid residual material was subjected to flash chromatography with silica-*H* as adsorbent (3 × 15 cm, $CHCl_3$/MeOH (10:1)). **19** was obtained with 21% yield (46 mg), **20** with 21% yield (46 mg) and **21** with 4% yield (9 mg) as a yellow powder.

1-(Carboxymethyl)uracil (*cis*−*syn*)-Cyclobutane Dimer Monobenzyl Ester Monoflavin Ester (19): mp 153−157 °C (dec); IR ($CHCl_3$) 3389 (w), 2989 (w), 1711 (s), 1650 (m), 1583 (m), 1550 (s), 1467 (m), 1406 (w), 1339 (w), 1267 (m, 1056 (w), 1017 (w), 922 (w) cm^{-1}; ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ 1.11 (t, $J = 7.1$ Hz, 3 H), 2.38 (s, 3 H), 2.49 (s, 3 H), 3.63 (m, 1 H), 3.71 (m, 1 H), 3.72 (d, $J = 17.4$ Hz, 1 H), 3.83 (d, $J = 17.4$ Hz, 1 H), 3.89 (q, $J = 7.1$ Hz, 2 H), 4.09 (d, $J = 17.4$ Hz, 1 H), 4.13 (d, $J = 17.4$ Hz, 1 H), 4.14 (m, 2 H), 4.52 (m, 2 H), 4.79 (m, 1 H), 5.03 (m, 1 H), 5.13 (s, 2 H), 7.35 (m, 5 H), 7.86 (s, 1 H), 7.91 (s, 1 H), 10.49 (s, 1 H), 10.57 (s, 1 H); ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 12.85, 18.73, 20.62, 35.95, 37.90, 38.45, 42.84, 46.96, 47.13, 54.80, 54.98, 61.44, 66.14, 116.23, 127.88 (2C), 128.12, 128.43 (2C), 130.96, 131.10, 134.06, 135.65, 136.00, 136.32, 146.79, 149.21, 152.57, 152.64, 154.76, 159.14, 166.86, 167.09, 168.55, 168.61; HRMS (FAB$^+$) m/z calcd for $C_{35}H_{35}N_8O_{10}$ ([M + H]$^+$) 727.2476, found 727.2527.

1-(Carboxymethyl)uracil (*trans*−*syn*)-Cyclobutane Dimer Monobenzyl Ester Monoflavin Ester (20): mp 201 °C; IR ($CHCl_3$) 3500 (w), 3389 (w), 3022 (w), 1706 (s), 1656 (m), 1600 (s), 1550 (s), 1467 (m), 1406 (w), 1356 (w), 1272 (m), 1056 (w), 972 (w), 922 (w), 850 (w) cm^{-1}; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 1.14 (t, $J = 7.1$ Hz, 3 H), 2.38 (s, 3 H), 2.50 (s, 3 H), 3.38 (m, 1 H),3.39 (m, 1 H), 3.89 (d, $J = 17.5$ Hz, 1 H), 3.90 (q, $J = 7.1$ Hz, 2 H), 3.99 (d, $J = 17.5$ Hz, 1 H), 4.09 (d, $J = 17.8$ Hz, 1 H), 4.13 (d, $J = 17.8$ Hz, 1 H), 4.29 (m, 1 H), 4.35 (m, 1 H), 4.44 (m, 1 H), 4.54 (m, 1 H), 4.91 (m, 2 H), 5.03 (d, $J = 12.4$ Hz, 1 H), 5.08 (d, $J = 12.5$ Hz, 1 H), 7.29 (m, 5 H), 7.86 (s, 1 H), 7.39 (s, 1 H), 10.65 (s, 1 H), 10.71 (s, 1 H); ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 12.78, 18.65, 20.52, 35.84, 38.23, 38.29, 38.91−40.00 (1C), 42.55, 48.10, 48.25, 59.08, 61.05, 65.96, 115.97,127.58, 127.73, 128.00, 128.26, 128.34, 130.86, 131.01, 133.92, 135.41, 135.86, 136.20, 146.69, 149.09, 151.44, 151.54, 154.51, 158.98, 169.13, 169.16, 169.56, 169.60; HRMS (FAB$^+$) m/z calcd for $C_{35}H_{35}N_8O_{10}$ ([M + H]$^+$) 727.2476, found 727.3066.

1-(Carboxymethyl)uracil (*trans*−*anti*)-Cyclobutane Dimer Monobenzyl Ester Monoflavin Ester (21): mp 173−175 °C (dec); IR ($CHCl_3$) 3378 (w), 3289 (w), 1744 (m), 1709 (s), 1672 (m), 1656 (m), 1583 (m), 1549 (s), 1500 (w), 1467 (m), 1406 (w), 1372 (m), 1267 (w) cm^{-1}; ^{1}H NMR (300 MHz, $(CD_3)_2SO$) δ 1.14 (t, $J = 6.9$ Hz, 3 H), 2.39 (s, 3 H), 2.50 (s, 3 H), 3.47 (m, 1 H), 3.76 (m, 1 H), 3.83 (d, $J = 17.5$ Hz, 1 H), 3.90 (q, $J = 6.9$ Hz, 2 H), 4.01 (d, $J = 17.5$ Hz, 1 H), 4.02 (d, $J = 17.8$ Hz, 1 H), 4.14 (m, 1 H), 4.22 (d, $J = 17.8$, 1 H), 4.35 (m, 1 H), 4.53 (m, 2 H), 4.94 (m, 2 H), 5.18 (m, 2 H), 7.38 (m, 5 H), 7.90 (s, 1 H), 7.91 (s, 1 H), 10.56 (s, 1 H), 10.69 (s, 1 H); MS (FAB$^+$) 727 (100, [M + H]$^+$).

1-(Carboxymethyl)uracil (*cis*−*syn*)-Cyclobutane Dimer Monopentyl Ester Monoflavin Ester (22) was synthesized following the general procedure described for the preparation of **19**−**21**. Instead of benzyl alcohol, 1-pentanol was used. **22** was obtained in 12% (23 mg) yield after chromatography with silica-*H*: mp 146−148 °C (dec); IR ($CHCl_3$) 3689 (w), 3389 (w), 1733 (m), 1711 (s), 1650 (m), 1583 (m), 1550 (s), 1467 (m), 1406 (w), 1339 (w), 1267 (m), 1017 (w), 922 (w) cm^{-1}; ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ 0.85 (t, $J = 7.0$ Hz, 3 H), 1.14 (t, $J = 7.0$ Hz, 3 H), 1.27 (m, 4 H), 1.56 (quint., $J = 7.0$ Hz, 2 H), 2.40 (s, 3 H), 2.50 (s, 3 H), 3.63 (m, 1 H), 3.71 (m, 1 H), 3.73 (d, $J = 17.4$ Hz, 1 H), 3.74 (d, $J = 17.4$ Hz, 1 H), 3.91 (q, $J = 7.1$ Hz, 2 H), 4.03 (m, 2 H), 4.06 (d, $J = 17.4$ Hz, 1 H), 4.10 (d, $J = 17.4$ Hz, 1 H), 4.14 (m, 2 H), 4.52 (m, 2 H), 4.81 (m, 1 H), 5.03 (m, 1 H), 7.87 (s, 1 H), 7.93 (s, 1 H), 10.50 (s, 1 H), 10.53 (s, 1 H); ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 12.84, 13.79, 18.73, 20.60, 21.69, 27.42, 27.68, 35.94, 37.95, 38.38, 42.82, 46.92, 47.14, 54.80, 54.91, 61.41, 64.66, 116.23, 130.94, 131.09, 134.04, 135.98, 136.33, 146.77, 149.22, 152.54, 152.60, 154.75, 159.12, 166.85, 167.06, 168.60, 168.61; HRMS (FAB$^+$) m/z calcd for $C_{33}H_{39}N_8O_{10}$ ([M + H]$^+$) 707.2789, found 707.2824.

1-(Carboxymethyl)uracil (*cis*−*syn*)-Cyclobutane Dimer Bisflavin Amide (2). The synthesis of **2** was achieved using the general procedure described for the preparation of **1, 17,** and **18**. Instead of the flavin alcohol **8**, the flavin amine **9** (100 mg, 0.286 mmol) was used. The reaction was stirred for only 5 h. The reaction mixture was worked up, and the residual, solidified material was not subjected to flash chromatography but recrystallized three times from methanol. **2** was obtained (60 mg, 44%) as a yellow powder: mp: 270−272 °C; IR (KBr) 3416 (m), 3322 (m), 1704 (s), 1650 (s), 1584 (s), 1547 (s), 1461 (s), 1337 (m), 1267 (m), 1230 (s), 1194 (m), 1172 (m), 1150 (m), 1018 (w), 928 (w), 845 (w), 807 (w), 770 (w) cm^{-1}; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 1.14 (t, $J = 6.6$ Hz, 6 H), 2.39 (s, 6 H), 2.49 (s, 6 H), 3.39 (d, $J = 16.7$ Hz, 2 H), 3.47 (m, 4 H), 3.62 (m, 2 H), 3.92 (q, $J = 6.6$ Hz, 4 H), 4.09 (m, 2 H), 4.11 (d, $J = 17.7$ Hz, 2 H), 4.63 (m, 4 H), 7.90 (s, 2 H), 7.94 (s, 2 H), 8.30 (m, 2 H), 10.43 (s, 2 H); ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 12.88, 18.72, 20.82, 35.66, 35.95, 38−42 (1C), 42.92, 48.08, 54.88, 115.99, 130.90, 131.07, 134.11, 135.93, 136.24, 146.75, 149.01, 152.37, 154.68, 159.13, 167.30, 168.36; HRMS (FAB$^+$) m/z calcd for $C_{44}H_{47}N_{14}O_{10}$ ([M + H]$^+$) 931.3599, found 931.3593.

1-(Carboxymethyl)uracil (*cis*−*syn*)-Cyclobutane Dimer Monopentyl Amide Monoflavin Amide (28). **28** was synthesized following the procedure for the preparation of **22**. The flavin amine **9** was used instead of the flavin alcohol **8**. The obtained crude orange oil was dissolved in MeOH and filtered to remove the bisflavin amide side product **2**. The obtained filtrate was subjected to flash chromatography with silica-*H* as the absorbent (3 × 20 cm, $CHCl_3$/MeOH (7:1 → 5:1)).

28 (51 mg, 27%) was obtained as a yellow powder: mp 197−199 °C (dec); IR ($CHCl_3$) 3333 (m), 3222 (m), 1706 (s), 1650 (m), 1583 (m), 1550 (s), 1467 (m), 1339 (w), 1272 (m), 1017 (w), 928 (w) cm^{-1}; ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ 0.85 (t, J = 7.0 Hz, 3 H), 1.14 (t, J = 7.0 Hz, 3 H), 1.24 (m, 4 H), 1.37 (quint., J = 7.0 Hz, 2 H), 2.41 (s, 3 H), 2.50 (s, 3 H), 3.02 (q, J = 7.0 Hz, 2 H), 3.34 (d, J = 17.4 Hz, 1 H), 3.44 (d, J = 17.4 Hz, 1 H), 3.49 (m, 2 H), 3.63 (m, 2 H), 3.92 (q, J = 7.1 Hz, 2 H), 4.01 (m, 1 H), 4.11 (m, 1 H), 4.12 (d, J = 16.5 Hz, 1 H), 4.13 (d, J = 16.5 Hz, 1 H), 4.58 (m, 1 H), 4.74 (m, 1 H), 7.90 (s, 1 H), 7.92 (t, J = 7.0 Hz, 1 H), 7.96 (s, 1 H), 8.25 (t, J = 7.0 Hz, 1 H), 10.39 (s, 1 H), 10.42 (s, 1 H); ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 12.89, 13.87, 18.74, 20.82, 21.79, 28.52, 28.75, 35.74, 35.96, 38.19, 38.51 (2C), 42.99, 48.08, 48.14, 54.49, 55.35, 116.04, 130.95, 131.09, 134.13, 135.95, 136.28, 146.75, 149.11, 152.39, 152.42, 154.73, 159.17, 166.95, 167.14, 167.55, 168.40; HRMS (FAB$^+$) m/z calcd for $C_{33}H_{41}N_{10}O_8$ ([M + H]$^+$) 705.3109, found 705.3126.

1-(Carboxymethyl)uracil Flavin Ester (3). 1-(Carboxymethyl)-uracil was synthesized according to the literature.[33] A solution of 1-(carboxymethyl)uracil (100 mg, 0.588 mmol) and (300 mg, 0.646 mmol) of BOP in 3 mL of DMF was stirred at room temperature for 10 min. After the addition of a solution of **8** (185 mg, 0.589 mmol) in DMF (3 mL), 10 drops of triethylamine were added and the reaction mixture was stirred for an additional 18 h at room temperature. The reaction mixture was diluted with 100 mL of water and extracted three times with 100 mL of $CHCl_3$. The combined organic layers were dried with $MgSO_4$, filtrated, and concentrated. The remaining orange oil was solidified through addition of diethyl ether. The residual material was subjected to flash chromatography with silica-*H* as adsorbent (3 × 15 cm, $CHCl_3$/MeOH (10:1)) to yield **3** (165 mg, 60%) as an orange powder: mp 190−192 °C; IR ($CHCl_3$) 1756 (w), 1694 (s), 1650 (m), 1583 (m), 1550 (s), 1461 (w), 1339 (w), 1189 (w) cm^{-1}; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 1.16 (t, J = 7.0, 3 H), 2.41 (s, 3 H), 2.50 (s, 3 H), 3.93 (q, J = 7.0, 2 H), 4.39 (s, 2 H), 4.58 (t, J = 5.3 Hz, 2 H), 4.94 (t, J = 5.3 Hz, 2 H), 5.00 (d, J = 7.9 Hz, 1 H), 7.51 (d, J = 7.9 Hz, 1 H), 7.87 (s, 1 H), 7.94 (s, 1 H), 11.29 (s, 1 H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 12.85, 18.73, 20.62, 35.94, 42.70, 48.27, 61.64, 101.03, 116.08, 130.95, 131.07, 134.03, 136.03, 136.20, 145.43, 146.87, 149.15, 150.77, 154.70, 159.08, 163.48, 168.11; MS (FAB$^+$) m/z 466 (100, [M + H]$^+$), 391 (13), 371 (28), 329 (11).

1-(Carboxymethyl)uracil Flavin Amide (4) was synthesized in analogy to **3** with the flavin amine **9** instead of the flavin alcohol **8**. **4** was obtained in 35% yield as an orange powder: mp >265 °C (dec); IR ($CHCl_3$) 1694 (s), 1650 (m), 1583 (m), 1550 (s), 1456 (w), 1339 (w), 1194 (m), 1118 (w), 1156 (w) cm^{-1}; ^{1}H NMR (400 MHz, $(CD_3)_2$-SO) δ 1.15 (t, J = 6.9, 3 H), 2.41 (s, 3 H), 2.49 (s, 3 H), 3.51 (q, J = 5.6 Hz,2 H), 3.93 (q, J = 6.9, 2 H), 4.20 (s, 2 H), 4.68 (t, J = 5.6 Hz, 2 H), 5.55 (d, J = 7.9 Hz, 1 H), 7.44 (d, J = 7.9 Hz, 1 H), 7.85 (s, 1 H), 7.95 (s, 1 H), 8.37 (t, J = 5.6 Hz, 1H), 11.25 (s, 1 H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 12.89, 18.75, 20.80, 35.97, 40.30, 42.91, 49.27, 100.70, 115.92, 130.93, 131.07, 134.14, 135.94, 136.25, 146.22, 146.79, 149.14, 150.91, 154.71, 159.17, 163.81, 167.78; MS (FAB$^+$) m/z 467 (37, [M + H]$^+$),447 (100), 391 (16), 371 (24).

Irradiation Experiments, Determination of the Quantum Yield, and Data Evaluation. Solutions (10^{-4} M) of the model compound in the corresponding solvents were prepared in quartz cuvettes (Hellma Type No.111-OS, 10 mm light path) equipped with a magnetic stirrer and a rubber septum. The solution was purged for 20 min with nitrogen. Then 25 μL (50 μL for diflavin compounds) of a sodium dithionite solution (50 mg in 5 mL aqueous buffer) was added. For reduction by catalytic hydrogenation, small amounts of reduction catalyst (Pd on Barium sulfate, Fluka) were added into the cuvette and the reaction mixture was stirred and purged first with nitrogen for 5 min, then with hydrogen for additional 20 min. Afterward the reaction mixture was allowed to stand under hydrogen at room temperature for another 5 min. The disappearance of the fluorescence of the solution (excitation at 366 nm, detection at 520 nm) was checked before and after the reduction to ensure complete reduction. For the pH-dependent measurements 0.5 M buffers were prepared. Citrate buffers were used for pHs 3, 4, 5, and 6, Tris·HCl buffers were employed for pHs 7, 8, and 9, and a phosphate buffer was used for pHs 10 and 11. Other substances added to the reaction mixtures such as acetic acid or triethylamine were added prior to the reduction. The irradiation of the cuvettes containing 3 mL samples was performed at 366 or 400 nm in a fluorescence spectrometer Spex 1680 equipped with a 0.22 m double-grating monochromator, a 450 W xenon lamp with a band-pass of 3.4 nm, and a magnetic stirrer in the sample compartment. All samples were stirred during the irradiation. During a series of measurements, the intensity of the light beam was repetitively determined by following the recommended procedures for potassium ferrioxalate actinometry.[44,45] The error in the number of light quants emitted from the lamp was determined to be ±10%. Separation of the photoproduct and the starting material was achieved on an analytical Lichrosphere (5/100, Merck) HPLC column (4 mm × 250 mm) by using 55% A and 45% B over 9 min to elute the photoproduct. Then, switching to 80% A and 20% B for additional 19 min caused the elution of the starting material (A, methanol; B, water). The peak areas were visualized and integrated by Knaur Eurochrom 2000 software. To eliminate inaccuracies of peak area stemming from fluctuations of the detection lamp, the determined peak areas of the photoproduct and the starting material were devided by the total peak area determined in the chromatogram (peak area photoproduct plus peak area starting material). The increase of photoproduct and the decrease of starting material were plotted against time and the obtained data fitted using a monoexponential decline or rise to maximum function with the program DeltaGraph Pro 3.5. To determine the experimental error of our measurements, the cleavage reaction of the model compound **28** in ethylene glycol with dithionite reduction was measured several times at different days. The obtained quantum yields were identical within an experimental error of ±8%.

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