Efficient Photosensitized Splitting of Thymine Dimer by a Covalently Linked Tryptophan in Solvents of High Polarity

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Tryptophan-thymine dimer model compounds used to mimic the repair reaction of DNA photolyase have been synthesized. The photosensitized cleavage of the dimer by the covalently linked tryptophan is strongly solvent-dependent with the reaction rates increasing in increasingly polar solvents, for example, the quantum yield $\Phi = 0.004$ in THF/hexane (5:95) and 0.093 in water. The fluorescence of the tryptophan residue is quenched by the dimer moiety by electron transfer from the excited tryptophan to the dimer. Fluorescence-quenching studies indicated that the electron transfer was efficient in polar solvents. The splitting efficiency of the dimer radical anion within the tryptophan$^{\bullet+}$–dimer$^{\bullet-}$ species is also remarkably solvent-dependent and increases with the polarity of the solvents. The back-electron-transfer reaction in the charge-separated species, which competes with cleavage, was suppressed in polar solvents. These results are in contrast to those of earlier solvent-dependent studies of indole-dimer systems, but they can be rationalized in terms of the differences in the distances between the chromophore unit and the attached dimer. The pH-dependent measurements of the splitting reaction and the deuterium isotope effect showed that the tryptophan radical cation within the charge-separated species does not deprotonate prior to the cleavage of the dimer radical anion.

Introduction

DNA repair has received heightened attention in recent years as ozone depletion threatens to significantly increase DNA damage by UVB radiation (280–320 nm).[1] One of the major effects on DNA of this type of radiation is the formation of cyclobutane pyrimidine dimers (CPD). DNA photolyases are flavin-containing repair enzymes that catalyze the efficient repair of *cis-syn* cyclobutane pyrimidine dimers by utilizing the energy of visible light to break the cyclobutane ring of the dimer. The mechanisms of CPD photolyases have been thoroughly investigated.[2,3] The enzymes contain a redox-active cofactor, flavin adenine dinucleotide (FAD), which operates in the fully reduced and deprotonated form ($FADH^-$), and an auxiliary antenna chromophore. A second cofactor activates the process of repair by absorbing near UV/Vis radiation (300–500 nm) and transferring the energy to the flavin. Subsequently, the excited $FADH^-$ transfers one electron to the cyclobutane pyrimidine dimer to form the dimer radical anion, which cleaves spontaneously, and then back-electron-transfer restores the dipyrimidine and the functional form of the flavin ready for a new catalytic cycle.

Several model compounds[4–6] that mimic the action of photolyase have been designed, for example, a chromophore attached to a pyrimidine dimer. Studies performed with these compounds have helped to unravel the mechanisms described in detail above. However, many questions concerning the physical-chemical mechanisms involved in the whole process remain unanswered.[3,7] In particular, it is unclear what factors cause the enzyme to mediate with a very high efficiency ($\Phi = 0.7$–0.98[3]) in DNA photoreactivation including the energy and electron transfer. In addition, under excitation with 280 nm light, Trp277, as a substrate binding site, can also directly repair CPD with a relatively high quantum yield ($\Phi = 0.56$) by electron transfer.[8,9]

Model systems appeared to be poorly efficient at repairing the lesion by intramolecular electron transfer, for example, $\Phi = 0.06$–0.40 for indole-dimer systems[10] and 0.062–0.016 for flavin-dimer systems[11] in various polar solvents such as water and 1,4-dioxane. This can be explained by the fact that model systems cannot stabilize the charge-separated species in which the electron is on the acceptor dimer before cleavage. Hence, competition between an unproductive back-electron-transfer reaction and the cleavage of the dimer radical anion is an important factor that leads to the low efficiency. In fact, rapid splitting of the CPD plays an important role in the highly efficient repair mediated by photolyase since it competes efficiently with the

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back-electron-transfer reaction. The splitting and back electron-transfer from a thymine radical anion to the flavin radical are expected to be complete within 0.5–2 ns,[12] whilst it takes 200[13] and 556 ns[14] for only the splitting process in solution. It is important to understand what factors can accelerate forward electron transfer from an excited chromophore to the linked dimer and suppress the back electron-transfer because these processes are undoubtedly counterparts in the enzymatic repair process.

In the chromophore-dimer systems, the photosensitized cleavage of the dimer by the attached indole,[10] arylamine[15] or methoxybenzene[16] was strongly solvent-dependent with efficient splitting taking place in solvents of low polarity. This unusual solvent effect can be explained by the fact that fast and unproductive charge recombination by back electron-transfer within the charge-separated species is suppressed in solvents of low polarity. The back electron-transfer in low-polar media would become so exothermic as to lie in the Marcus inverted region, which would significantly reduce the rate of the transfer reaction.[10,15,16] The results seem to support previous findings that the photolyase may provide a hydrophobic environment for the substrate (dimer).[8,17]

However, an inverse solvent dependence was observed in covalently linked flavin-dimer systems by Carell and coworkers.[11,18] By taking two sets of measurements in water/ethylene glycol mixtures and various organic solvents, they observed increased splitting efficiencies in polar solvents. In contrast to the non-flavin-containing systems, which yield a zwitterionic intermediate after photoinduced electron transfer, the reduced and deprotonated flavin systems result in a non-zwitterionic intermediate as a result of a shift of the negative charge from the flavin moiety to the dimer. Since a much smaller driving force can be expected for the unproductive back charge transfer, it shouldn't lie in the Marcus inverted region. The results suggested that an unusually polar flavin-containing pocket, observed in the X-ray crystal structures of the *E. coli* photolyase, may be required to increase the catalytic repair efficiency.[11]

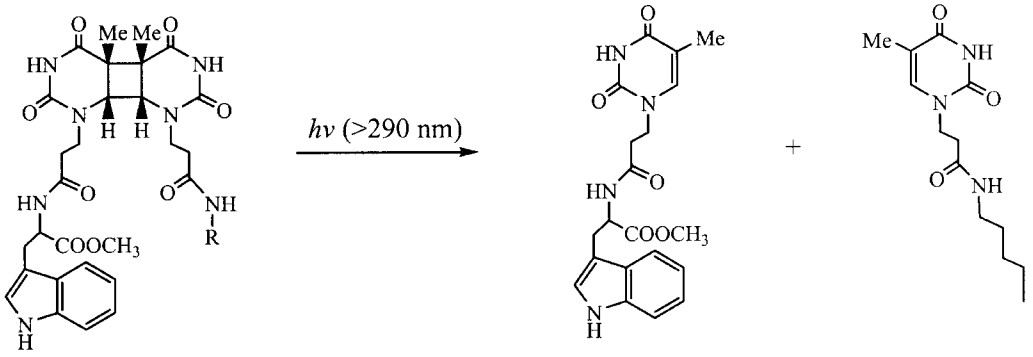
In this work, we have prepared covalently linked tryptophan-dimer model compounds **1** and **2**, and investigated the photosensitized splitting of the thymine dimer by the attached tryptophan in various solvents (Scheme 1). The quantum yield for the splitting of the dimer is very sensitive to the polarity of the solvents. Remarkably high splitting efficiencies were observed in solvents of high polarity. Interestingly, this solvent effect contrasts strongly with the results[10,15,16] obtained previously with non-flavin-dimer systems, but is in agreement with the results[11,18] obtained with flavin-dimer systems. Furthermore, these results together with data from fluorescence quenching experiments and studies of the effect of pH on splitting of the dimer, some new insights into the intramolecular electron-transfer process in the tryptophan-dimer system were gained.

Results

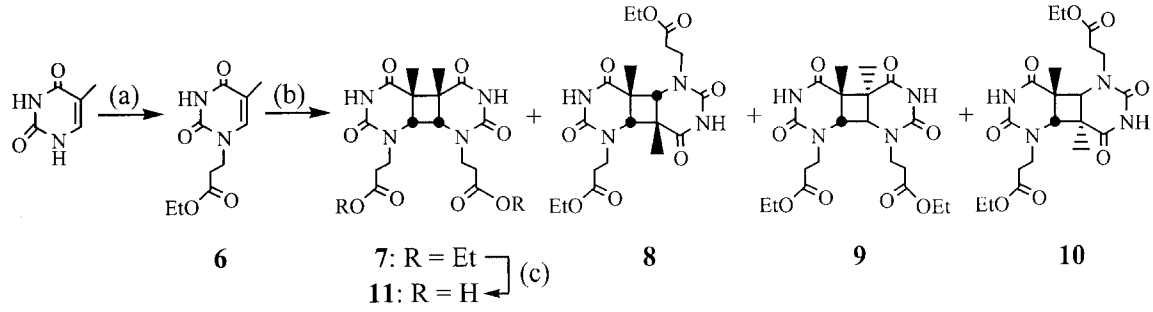
Synthesis of the Model Compounds 1 and 2

The preparation of the *cis-syn* thymine dimer (Scheme 2) is a prerequisite for the synthesis of the model compounds. The ethyl ester **6** of 1-(2-carboxyethyl)thymine was prepared from thymine.[19] In the presence of acetone, an acetonitrile solution of **6** was placed in a Pyrex photoreactor ($\lambda >$ 290 nm); pure N_2 was bubbled through the solution which was irradiated with a 300-W high-pressure Hg lamp to give a mixture of all four possible isomers. The *cis-syn* thymine dimer **7** was separated by chromatography on silica gel-*H*. X-ray analysis of the *cis-syn* isomer allowed the correct structural assignment (Figure 1). Subsequent hydrogenolytic cleavage of the ethyl ester gave the *cis-syn* thymine dimer dicarboxylic acid **11** as a white powder in excellent yield. The tryptophan methyl ester **5** was prepared from tryptophan and methanol in 91% yield.

The model compounds **1** and **2** were prepared by condensation of tryptophan methyl ester (**5**) and the thymine dimer dicarboxylic acid **11** with (benzotriazol-1-yl)oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP)[20] activation of the carboxylic acids in DMF (Scheme 3). After addition of one equivalent of the tryptophan methyl ester, the mixture was stirred for 2 h. A large excess of *n*-pentylamine was then added and allowed to react for 5 h



Scheme 1.



Scheme 2. Reagents and conditions: (a) ethyl acrylate, hydroquinone, NaOH, EtOH, reflux, 20 h; (b) *hv* (> 290 nm), acetone/MeCN, room temp., 20 h; (c) 5 M HCl, reflux, 1 h.

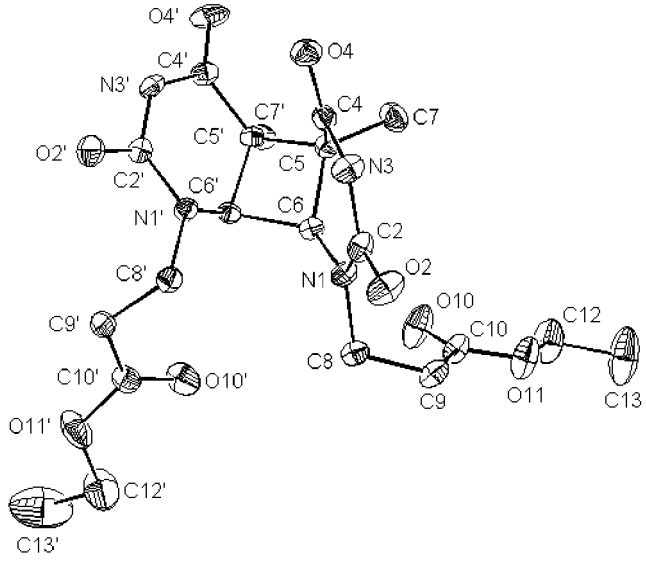
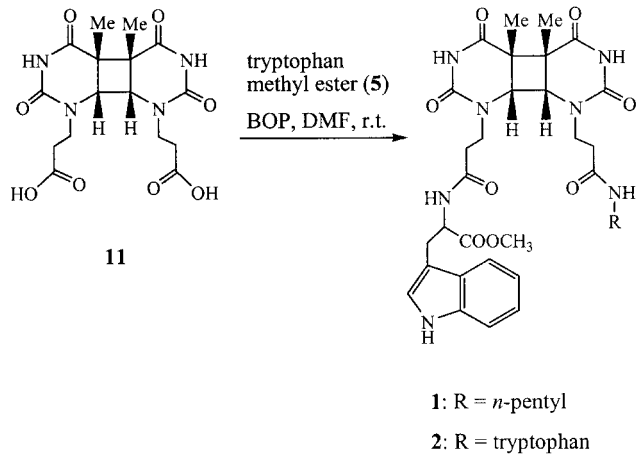


Figure 1. X-ray crystal structure of the *cis-syn* thymine photodimer **7** shown with 30 % ellipsoids. Crystal data for **7**: colorless needles, $C_{20}H_{28}N_4O_8$, M = 452.46, monoclinic, space group $P21/n$, D_c = 1.342 g cm^{-1}, Z = 4, a = 8.427(3), b = 12.346(5), c = 21.542(8) Å, β = 92.104(7)°, V = 2239.7(15) Å^3, T = 293(2) K, μ(MoK_α) = 0.105 mm^{-1}, 11202 reflections collected, 3935 unique (R_{int} = 0.0543), R_1 = 0.0563, wR_2 = 0.1241 [$I > \sigma 2(I)$]. CCDC-253225 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.



Scheme 3.

to yield compound **1**. More than two equivalents of the tryptophan methyl ester were added and allowed to react for 5 h to afford compound **2**. Both the model compounds **1** and **2** were extracted with chloroform and then purified by chromatography on silica gel-*H* to give white powers in 21 and 25% yields, respectively.

Photosplitting Properties of the Model Compounds

The model compound **1** or **2** in aqueous solution was irradiated with a 290 nm light beam from a fluorescence spectrometer containing a 125-W Xe lamp. Analysis of the photolyzed mixture by reversed-phase HPLC and co-injection of the synthesized expected photoproducts **3** and **4** confirmed that the model compounds react cleanly to give only **3** from **2** and only **3** and **4** from **1**. Furthermore, the photoproducts **3** and **4** were isolated and identified by NMR spectroscopy. A representative set of HPLC chromatograms showing the simultaneous splitting of compound **1** into **3** and **4** is depicted in Figure 2. Clearly, the reaction of compound **1**, with a retention time of 5.3 min, to the photosplit products **3** and **4**, with retention times of 3.3 and 3.5 min, respectively, is a clean conversion as no other products could be detected.

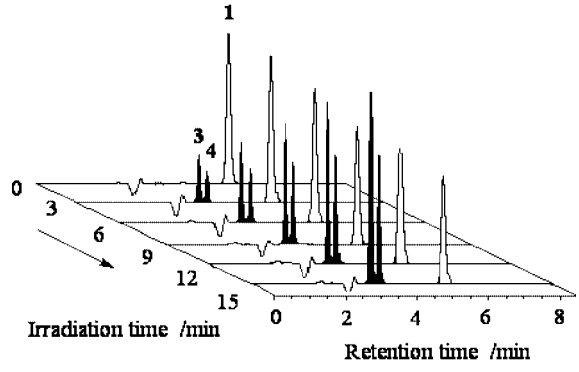


Figure 2. Typical HPLC chromatograms obtained after 0, 3, 6, 9, 12 and 15 min of irradiation of **1** in water (3 mL, 5×10^{-5} M) with 290 nm light. Assay conditions: C_{18} reversed-phase column, 70% methanol and 30% water as eluent, detection at 270 nm. Retention times: 3.3 min (product **3**), 3.5 min (product **4**), 5.3 min (model compound **1**).

The splitting reaction occurs by intramolecular electron transfer since the quantum yield is not concentration-dependent at 0.01, 0.05 and 0.1 mM within an experimental error of ±5%. This ruled out the possibility of intermolecular photosensitization between one molecule of the model compound in the excited state and another molecule compound in the ground state, that is, the excited tryptophan unit of one model molecule being responsible for the cleavage of the dimer unit of another model molecule.

Quantum Yields of Splitting of 1 and 2

To measure the quantum yields for the splitting of the model compounds Φ [Φ = (rate of dimer splitting)/(rate of photons absorbed)], all sample solutions ($\approx 5\times10^{-5}$ M, 3 mL) were prepared in the corresponding solvents and placed in quartz cuvettes (10 × 10 mm) with a Teflon stopper and then irradiated with 295 nm light from a fluorescence spectrometer. By measuring the increase in the absorbance at 273 nm (ΔA_{273}) after certain intervals of time, a rate for the dimer splitting was obtained from the straight line resulting from a plot of ΔA_{273} against irradiation time. The intensity of the excitation light beam was measured by ferrioxalate actinometry.[21] From the intensity of the light beam and the absorbance of the sample at 295 nm before irradiation, the rate of photon absorption was calculated. Based on these results, the quantum yields for the dimer splitting of **1** and **2** were calculated and the values are listed in Table 1.

The data show that the quantum yields for the splitting of **1** and **2** are strongly solvent-dependent. The values of Φ decrease with solvents in the order water > alcohols > dipolar aprotics > nonpolar, ranging from 0.093 in H_2O to 0.004 in THF/hexane (5:95) for **1**, and 0.079 in H_2O to 0.003 in THF/hexane (5:95) for **2**. The order of the solvents reflects their ability to solvate and stabilize charge. Hence, the efficiency of dimer splitting is enhanced in solvents of high polarity.

Furthermore, the solvent dependence of Φ was measured in solutions of water/THF in various ratios (Table 2). A gradual decrease in the quantum yield was observed upon addition of increasing amounts of THF and the lowest value of Φ was obtained in pure THF. The systematic reduction in solvent polarity results in a significant decrease in the quantum yield for the splitting reaction of compound **1**, ranging from 0.092 in water/THF (80:20) to 0.050 in water/THF (5:95). This further demonstrates that efficient splitting occurs in solvents of high polarity.

Table 2. Splitting efficiencies for compounds **1** and **2** in water/THF binary solvent.

Solvent mixture (THF:H_2O, v/v)	Compound **1**		Compound **2**	
	Φ	Q	φ_{spl}	Φ
100:0	0.016	0.36	0.045	0.021
95:5	0.050	0.54	0.092	0.054
90:10	0.059	0.65	0.091	0.057
80:20	0.069	0.70	0.098	0.062
60:40	0.077	0.73	0.105	0.070
50:50	0.091	0.75	0.121	0.076
40:60	0.091	0.78	0.117	0.078
20:80	0.092	0.80	0.115	0.078
0:100	0.093	0.81	0.115	0.079

The quantum yields of compound **2** are lower than those of compound **1** in polar solvents, and similar in low-polar solvents. Hydrophobic indoles can often form π-stacked aggregates particularly in polar solvents. Hence, the lower quantum yields for compound **2** may result from intramolecular tryptophan–tryptophan stacking which inhibits the electron-transfer reaction in polar solvents. Small differences in the UV absorption spectra of compounds **1** and **2** were observed in water, but not in THF, which supports this explanation.

Fluorescence Emission of 1 and 2

Figure 3A shows the fluorescence emissions of **1** and **2** (symbolized as TrpH–D) as well as the fluorescence emission of the free tryptophan methyl ester **5** (TrpH, H denotes the N1 proton) in water at room temperature. The corresponding results for **1**, **2** and **5** in THF are shown in Figure 3B. It can be seen that the fluorescence emission of the tryptophan unit in **1** and **2** are quenched by the covalently linked thymine dimer and that the extent of fluorescence quenching [Equation (1), where F is the fluorescence intensity] is highly sensitive to the polarity of the solvent.

Table 1. Dependence of splitting efficiency on solvent for compounds **1** and **2**.

Solvents[a]	Compound **1**			Compound **2**		
	Φ[b]	Q	φ_{spl}	Φ[b]	Q	φ_{spl}
Water	0.093	0.81	0.115	0.079	0.76	0.104
Acetonitrile	0.041	0.48	0.085	0.036	0.46	0.078
Methanol	0.072	0.65	0.111	0.057	0.50	0.114
Ethanol	0.070	0.61	0.115	0.062	0.52	0.119
Propanol	0.066	0.53	0.125	0.055	0.46	0.120
2-Propanol	0.063	0.48	0.131	0.041	0.45	0.091
THF	0.016	0.36	0.044	0.021	0.32	0.066
Ethyl acetate	0.014	0.49	0.029	0.012	0.39	0.031
1,4-Dioxane	0.013	0.41	0.032	0.016	0.50	0.032
THF/hexane (5:95)	0.004	0.27	0.015	0.003	0.34	0.009

[a] Arranged in order of decreasing dielectric constant. [b] Average of two determinations; 10 nm bandwidth.

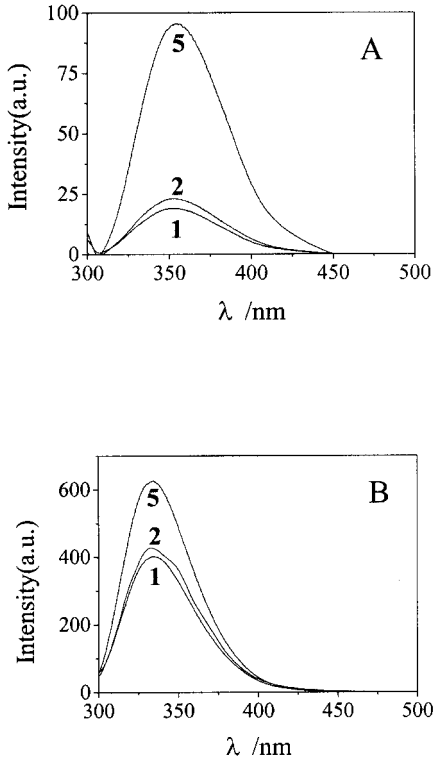


Figure 3. Relative fluorescence emission intensities of tryptophan methyl ester **5** and the model compounds **1** and **2** in water (A) and in THF (B) upon excitation at 290 nm.

$$Q = 1 - (F_{TrpH-D}/F_{TrpH}) \quad (1)$$

The values of Q increase with increasing polarity of the solvents (Table 1). The fluorescence quenching of the tryptophan unit in **1** or **2** is not a result of significant absorption of exciting light by the dimer in **1** or **2**. As the molar extinction coefficient of the thymine dimer **7** is much less than that of the tryptophan methyl ester **5** at 290 nm, for example, in water $\varepsilon_{290} = 40\ M^{-1}cm^{-1}$ for **7** and $5.4\times10^3\ M^{-1}cm^{-1}$ for **5**, an internal filter effect should not be significant.

Fluorescence quenching is probably a consequence of intramolecular electron transfer from the excited tryptophan to the linked dimer. Since there is almost no overlap between the emission spectra of the tryptophan methyl ester **5** and the thymine dimer **7** (not shown), singlet–singlet energy transfer is an improbable mode of fluorescence quenching in the model compounds. Hence, electron transfer should be the predominant mode of fluorescence quenching in **1** and **2**. The data in Table 1 show that electron transfer from excited tryptophan to the linked dimer becomes more efficient in more polar solvents. This implies that the formation of the charge-separated species would reasonably be expected to be accelerated in strongly polar media, which would allow decay processes caused by electron transfer to predominate, and fluorescence and nonradiative processes to be suppressed.

Effect of pH on the Splitting Reaction

The electron-transfer reaction from the excited tryptophan residue to the attached dimer gives a zwitterionic intermediate $TrpH^{\cdot+}$–$D^{\cdot-}$, which has a high driving force for charge recombination and therefore is a very short-lived intermediate. Since the pK_a for the deprotonation at the N1 site of the indole in the tryptophan radical cation ($TrpH^{\cdot+}$) is about 4,[22] it will deprotonate in neutral aqueous solution to give a neutral radical ($Trp^{\cdot}$). If the deprotonation of the tryptophan radical cation within $TrpH^{\cdot+}$–$D^{\cdot-}$ occurs prior to the cleavage of the dimer radical anion, a non-zwitterionic intermediate $Trp^{\cdot}$–$D^{\cdot-}$ would form [Equation (2)] that should have a longer lifetime, consequently yielding a more efficient splitting.

$$TrpH^{\cdot+}\text{–}D^{\cdot-} \overset{pK_a}{\rightleftharpoons} Trp^{\cdot}\text{–}D^{\cdot-} + H^+ \quad (2)$$

To examine whether the deprotonation of $TrpH^{\cdot+}$–$D^{\cdot-}$ does occur prior to the cleavage, we investigated the effect of pH on the splitting reaction. The quantum yields for the photosplitting of **1** and **2** on irradiation with 295 nm light in solvent mixtures of water/THF (80:20) buffered over the pH range of 1 to 13 is shown in Figure 4. The pH-dependent measurements show small changes from pH 3 to 10. No notable change in Φ was observed between pH 3 and 5. At pH < 3, both the dimer and the tryptophan are possibly protonated, which results in a slight decrease in the values of Φ. From the UV spectra of the model compounds in solutions at various pH, a new absorption peak at about 240 nm appears at pH 10–11 (inset of Figure 4), which may be indicative of deprotonation of the dimer since the pK_a of the deprotonation of the cyclobutane uracil dimer is 10.7.[23] Thus, electron transfer to a dimer anion would be inhibited when pH > pK_a.

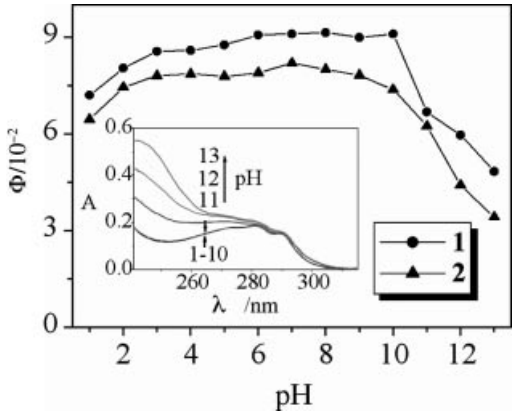


Figure 4. Investigation of effect of pH on the splitting efficiency of **1** and **2** in water/THF (80:20) solutions with 0.1 M buffer. Inset: UV absorption spectra of 3×10^{-5} M**1** in water/THF (80:20) solutions at pH 1–13.

Because breaking a N–D bond requires more energy than a N–H bond, the deprotonation ($K_a = [H^+][Trp^{\cdot}]/$

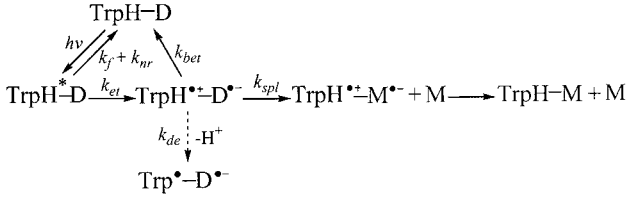
[$TrpH^{\cdot+}$]) of the tryptophanal radical cation in D_2O would be inhibited, that is, the equilibrium in D_2O would be shifted towards the protonated state to give the neutral tryptophan radical Trp· in a lower concentration than in H_2O.

If the deprotonation occurs prior to the cleavage of dimer radical anion in $TrpH^{\cdot+}$–$D^{\cdot-}$, the concentration of Trp·–$D^{\cdot-}$ in D_2O will be lower than that in H_2O. Thus, the value of Φ would be lower in D_2O than in H_2O, that is, a deuterium isotope effect on the splitting of the dimer would be observed. Hence, an experiment to measure the deuterium isotope effect was performed to examine whether the N1–H(D) bond of indole breaks prior to cleavage. Under the same irradiation conditions, however, there were no differences in the splitting rates of compound **1** in H_2O and D_2O within experimental error, that is, the bond breaking of the N1–H(D) did not occur before cleavage of the dimer.

Therefore, the above results show that deprotonation of the tryptophan radical cation within $TrpH^{\cdot+}$–$D^{\cdot-}$ is slower than both the splitting of the dimer radical anion and the charge recombination of $TrpH^{\cdot+}$–$D^{\cdot-}$.

Discussion

The photophysical and photochemical processes of the model compounds are illuminated by the simple mechanism shown in Scheme 4. Upon irradiation with light of $\lambda >$ 290 nm, a tryptophan unit in the model compound absorbs a photon to produce an excited state of the tryptophan. The excited state has the following relaxation pathways: fluorescence (k_f), internal conversion and intersystem crossing (together represented by k_{nr}), and electron transfer to the related dimer (k_{et}). The sum of the efficiencies of these processes is 1, that is, $\varphi_f + \varphi_{nr} + \varphi_{et} = 1$. The charge-separated species ($TrpH^{\cdot+}$–$D^{\cdot-}$) formed by electron transfer can undergo two processes, splitting (φ_{spl}) or back electron-transfer (φ_{bet}), which results in an unproductive reversal, and their efficiencies add up to 1. There are two pairs of competitions in these processes: 1) the photophysical process (k_f and k_{nr}) and electron transfer (k_{et}) and 2) the splitting (k_{spl}) and back electron-transfer (k_{bet}). In the two pairs of competitions, k_{et} and k_{spl} contribute to the observed quantum yield of dimer splitting. The relative quantum efficiencies of these processes determine the observed quantum yield of dimer splitting, $\Phi = \varphi_{et}\varphi_{spl}$.



Scheme 4.

The observed quantum yield for dimer splitting reflects the efficiency of the overall photosensitized cycloreversion of the dimer by the linked tryptophan. The variations in the quantum yields for dimer splitting and in the fluorescence quenching (Q) with solvent polarity allow the effect of solvent on the two pairs of competitions to be evaluated.

The efficiency of electron transfer can be discussed in terms of the following reasonable assumption.[16] The rate constants for fluorescence and the nonradiative relaxation pathways are unaltered by attachment of the dimer to the tryptophan [i.e., $k'_f(\mathbf{5}) = k_f(\mathbf{1}$ or $\mathbf{2})$ and $k'_{nr}(\mathbf{5}) = k_{nr}(\mathbf{1}$ or $\mathbf{2})$] and electron transfer (k_{et}) can occur in the excited model species. On this basis and from Equation (1), Equation (3), Equation (4)and Equation (4), it was deduced that the efficiency of electron transfer is equal to the degree of fluorescence quenching, that is, $\varphi_{et} = Q$.

$$F_{TrpH-D} \propto k_f/(k_f + k_{nr} + k_{et}) \quad (3)$$

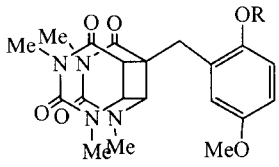
$$F_{TrpH} \propto k'_f/(k'_f + k'_{nr}) \quad (4)$$

$$\varphi_{et}=k_{et}/(k_f + k_{nr} + k_{et}) \quad (5)$$

Thus, values of φ_{spl} were calculated from $\varphi_{spl} = \Phi/Q$ and are listed in Table 1 and Table 2. It is evident that the splitting efficiencies of the dimer radical anion are remarkably dependent on the solvent polarity. On the basis of the values of Q and φ_{spl} in Table 1 and Table 2, it might reasonably be expected that electron transfer k_{et} and splitting k_{spl} are accelerated in solvents of high polarity, or that fluorescence and back electron-transfer are suppressed.

This solvent dependence contrasts with the results of earlier work[10,15,16] in which the solvent dependence of the splitting reaction was investigated in detail by Rose and coworkers. In their work, the splitting efficiency of the model compounds was found to be rather strongly solvent-dependent and increased with decreasing solvent polarity, for example, in 1,4-dioxane Φ = 0.2,[10] 0.4[15] and 0.24[16] and in water Φ = 0.06,[10] 0.02[15] and 0.05.[16] The results can be explained in terms of the competition between the splitting and back electron-transfer within the charge-separated intermediate. Since low-polar media strongly destabilize the intermediate, charge recombination by back electron-transfer becomes so exothermic as to lie in the Marcus inverted region,[24] which would significantly reduce the rate of the transfer reaction. In the methoxybenzene-containing model compounds (**12**[16]), the quantum yields for the dimer splitting are higher and the degree of fluorescence quenching is lower (i.e. φ_{et} is lower) in solvents with lower polarity. Thus, the quantum yields calculated for the dimer radical anion φ_{spl} are higher in low-polar media.[16] This implies that solvents of low polarity are unfavorable for electron transfer, but favor the cleavage of the dimer radical anion by suppressing back electron-transfer.

However, the solvent dependence observed in our work is in accord with conclusions drawn from studies of flavin-dimer model compounds.[11,18] In contrast to the strong solvent dependence, the splitting reaction in flavin-dimer systems was found to have a rather low total solvent depen-



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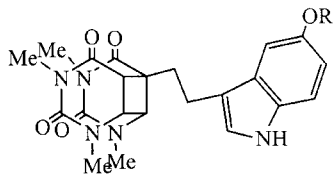
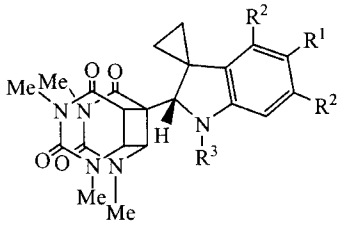
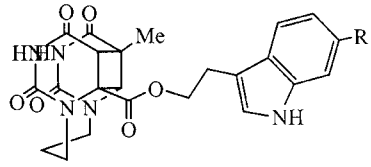


13[10]



14[15]



15[25]

dence. This weak solvent dependence was demonstrated by the formation of a non-zwitterionic intermediate, $FlH^{\cdot}$–Dimer$^{\cdot-}$, which was formed by a shift in charge in the FlH^{-}–Dimer species.[11,18]

This difference in solvent behavior may be due to differences in the distances (spacers) between the electron acceptor (dimer) and donor (chromophore) in the model compounds, for example, the 1,3-dimethyluracil dimers **12**,[16]**13**[10] and **14**[15] have relatively short spacers and the thymine dimers **1** and **2** have long spacers. The solvent reorganization energy (λ_s) involved in moving an electron from the donor to the acceptor without rearranging the solvation shell can be determined from Equation (6),

$$\lambda_s = e^2[(2r_D)^{-1} + (2r_A)^{-1} - (R_{DA})^{-1}](\varepsilon_{op}^{-1} - \varepsilon_s^{-1}) \quad (6)$$

where ε_{op} and ε_s are the high frequency and static dielectric constants of the solvent, respectively, r_D and r_A are donor and acceptor radii and R_{DA} is the center-to-center distance between the ions. Because of the very short spacers (a small R_{DA}) for **12**, **13** and **14**, the solvent reorganization energy for charge recombination for the chromophore$^{\cdot+}$–dimer$^{\cdot-}$ species is small. Although $-\Delta G°$, which can be determined from Equation (7),

$$-\Delta G° = E_{D^{\cdot+}/D} - E_{A/A^{\cdot-}} - e^2/\varepsilon r \quad (7)$$

where $E_{D^{\cdot+}/D}$ and $E_{A/A^{\cdot-}}$ are potentials for one-electron oxidation of the donor and reduction of the acceptor, respectively, and ε and r are the static dielectric constant of the solvent and the R_{DA} in Equation (6), respectively, would also decrease, this decrease is less than λ_s.

As the polarity of the solvent decreases, such that $\lambda_s < -\Delta G°$, the back-electron-transfer reaction enters the Marcus inverted region[24] in which the rate constant for the back electron-transfer (k_{bet}) is reduced [Equation (8), where k_B is Boltzmann's constant] and leads to an efficient splitting.[10,15,16]

$$k_{bet} = A' \exp[-\Delta G° + \lambda_s)^2/4\lambda_s k_B T] \quad (8)$$

In contrast, the solvent reorganization energy for our model compounds with a relatively long spacer (a large R_{DA}) is higher, such that $\lambda_s > -\Delta G°$. As the polarity of the solvent decreases, the back-electron-transfer reaction wouldn't enter the Marcus inverted region. In the Marcus normal region ($\lambda_s > -\Delta G°$), the zwitterionic intermediate can be stabilized by high-polar media and suppress the unproductive charge recombination process, k_{bet}, to result in efficient splitting.

In another indole-dimer system, **15**, which has a longer spacer and a dimer derived from thymine and orotic acid,[25] a more efficient splitting reaction occurred in water (Φ = 0.081) than in ethanol (Φ = 0.068). The corresponding values of Φ were 0.06 and 0.24 for **13**[10] and 0.02 and 0.12 for **14**[15] in the two solvents, respectively. Therefore, the length of spacer may play an important role in both the splitting efficiency and the solvent dependence of splitting of the chromophore-dimer model compounds.

The X-ray crystal structures of *E. coli* photolyase revealed a hole, the putative substrate-binding site, which has the right dimensions to fit the pyrimidine dimer, and the residues lining its sides are hydrophobic on one side and polar on the other.[7] This asymmetry was suggested to fit well with the asymmetric polarity of a pyrimidine dimer in which the cyclobutane ring is hydrophobic and the opposite edges of the dimer have oxygen and nitrogen atoms capable of forming hydrogen bonds.[7] This specific hole may contribute to the accelerating cleavage of the dimer radical anion and to the suppression of the unproductive back electron-transfer.

The splitting efficiencies in solutions of various pH and deuterium isotope experiments showed that Trp$H^{\cdot+}$–$D^{\cdot-}$

does not deprotonate prior to the cleavage of the dimer radical anion (Scheme 4), that is, $k_{de} < k_{spl}$. The tryptophan radical cation would deprotonate at N1 to form a neutral radical in neutral solution. The process is completed within about 1 μs, and in >100 μs in acidic solutions at pH 3–4,[26] and in 300 ns for the radical cation of the tryptophan residue ($^{306}TrpH^{\cdot+}$) in photolyase.[27] The first two figures show that the deprotonation rate is slower than the rate for the splitting of the dimer radical anion (200 ns[13] and 556 ns[14]). In alkaline solution, the tryptophan radical cation was short-lived,[26] thus it is possible that the deprotonation process was more favourable than the splitting of the dimer radical anion. However, the dimer will also deprotonate at N3 in alkaline solution at pH > 10 which would inhibit electron transfer to the dimer anion. Because the values of φ_{spl} are much less than 1 (Table 1 and Table 2), $\varphi_{spl} + \varphi_{bet} = 1$, the back-electron-transfer reaction should dominate over dimer splitting in the $TrpH^{\cdot+}$–$D^{\cdot-}$ species, that is, $k_{spl} < k_{bet}$. Hence, the rate constants would increase in the order $k_{de} < k_{spl} < k_{bet}$.

Conclusions

In summary, by using synthesized model compounds **1** and **2** to mimic the enzymatic repair reaction, the photosensitized splitting reaction of the *cis-syn* thymine dimer by covalently linked tryptophan to give photoproducts **3** and **4** was confirmed to be a clean conversion upon irradiation with light of $\lambda > 290$ nm.

The splitting reaction is strongly solvent-dependent and the splitting efficiencies increase in increasingly polar solvents. Fluorescence quenching of the tryptophan unit by the attached dimer shows that efficient electron transfer from the excited tryptophan unit to the dimer occurs in polar solvents. This result is in agreement with the observation from non-indole-dimer model compounds (**12**[16]). Furthermore, the quantum yields of the dimer radical anion exhibit the same solvent dependence. Thus, back electron-transfer, which competes with the cleavage of the dimer radical anion, is suppressed. Therefore, solvents of high polarity enhance the efficiency of the forward electron transfer from the excited tryptophan moiety to the dimer and suppress the unproductive back electron-transfer (charge recombination) thereby allowing the model compounds to undergo highly efficient splitting reactions.

The solvent dependence of the splitting efficiencies observed in this work contrasts with earlier solvent-dependent studies.[10,15,16] These differences in solvent dependence may be a result of the different lengths of spacers between the donor and acceptor in the model compounds. Compared with model systems[10,15,16] with short spacers, the solvent reorganization energy for the back electron-transfer within charge-separated species with longer spacers is higher and does not lie in the Marcus inverted region.

The solvent dependence in our model systems, which supports conclusions[11,18] drawn from studies of flavin-dimer model compounds, implies that the polar flavin-containing pocket observed in the X-ray crystal structure of *E. coli.* photolyase may be required to increase the efficiency of catalytic repair.

The pH-dependent measurements show that deprotonation of the tryptophan radical cation after photoinduced electron transfer is slow and doesn't occur prior to cleavage. As the splitting and back electron-transfer after the splitting are expected to be complete within 0.5–2 ns,[12] the splitting process in the enzyme is much faster than that in solution, 5×10^6 s^{-1}.[14] Hence, when the tryptophan residue directly repairs the substrate with high efficiency ($\Phi = 0.56$) upon irradiation with light of $\lambda = 280$ nm,[9] $^{277}TrpH^{\cdot+}$ would not be expected to deprotonate before splitting of the dimer radical anion. This fast splitting of the dimer radical anion in the enzyme, which competes with the unproductive back electron-transfer, should be a very important factor for high enzymatic repair efficiency.

Experimental Section

General Remarks: All materials were obtained from commercial suppliers and used without further purification. Solvents of technical quality were distilled prior to use. DMF was dried overnight with $MgSO_4$ and was distilled after removal of anhydrous $MgSO_4$. Ethanol, propanol, 2-propanol and ethyl acetate were dried overnight with K_2CO_3 and were distilled after removal of the K_2CO_3. Water was deionized and then double distilled. Acetonitrile and methanol were spectroscopic grade from commercial suppliers and were used without further purification. Tetrahydrofuran and 1,4-dioxane were distilled from sodium metal. ^1H and ^{13}C NMR spectra were measured with a Bruker AV 300 spectrometer operating at 300 and 75 MHz, respectively. FTIR spectroscopy was measured with a Bruker Vector22 Intrared Spectrometer. Mass spectra were obtained with a Micromass GCF TOF mass spectrometer. Elemental analyses were measured with an Elemental Vario EL III spectrometer.

Measurement of the Quantum Yield for Splitting: To measure the quantum yield for the splitting of the model compounds, samples (3 mL) were irradiated with 295 nm light from a 970 CRT fluorescence spectrometer (Shanghai Analysis Instrument) containing a 125-W Xe lamp and a monochromator with a 10-nm slit. To measure the extent of dimer splitting the increase in the absorbance at approximately 273 nm due to the regeneration of the 5,6-double bonds of the pyrimidine units was monitored. After certain intervals of time, the absorbance of the irradiated solutions was recorded with a Lambda 45 UV/Vis spectrometer (Perkin-Elmer Instruments). Before each UV spectrum could be recorded a short period of time (about 2 min) after irradiation was required in order to obtain a homogeneous irradiated mixture. Potassium ferrioxalate actinometry[21] was performed in triplicate before and after sample irradiation to measure the intensity of irradiation (295 nm). Within an experimental error of ±5%, the quantum yields for splitting were constant irrespective of whether or not N_2 was passed through solutions prior to irradiation. The values of Φ for **1** in three representative aerated and N_2-saturated solvents (in parentheses) are as follows: H_2O, 0.093 (0.091); propanol, 0.066 (0.065); and THF, 0.016 (0.017). Hence, aerated solutions were employed in all quantum yield measurements unless otherwise noted.

The absorbances at 273 (A_{273}) and 295 nm (A_{295}) were measured at certain intervals of time after irradiation. The change in A_{273} (ΔA_{273}) of the solution depends on the extent of splitting of the

model compounds. As the model compounds fully split to form products **3** and **4**, the changes in the molar extinction coefficients ($\Delta\varepsilon_{273}$) were obtained from the UV absorption spectra of **1** and **2** and the splitting products **3** and **4**; the value of $\Delta\varepsilon_{273}$ employed was 1.92×10^4 M^{-1} cm^{-1}. The splitting concentration (c_{spl}) of the model compound was obtained from $\Delta A_{273}/\Delta\varepsilon_{273}$.

The percentage of splitting of the model compounds was kept within 5% in all measurements of the quantum yields. A plot of c_{spl} against the irradiation time (t/min) fits a straight line well (all correlation coefficients, R, > 0.996). The slope B of the straight line gives the rate of splitting of the model compounds. The intensity of the incident light I_0 (unit: Einstein min^{-1}) was measured by using ferrioxalate actinometry.[21] The intensity of the light absorbed (I_a) by a solution was calculated from Beer's law, $I_a = I_0(1 - 10^{-A295})$. From these values the quantum yields could be calculated: $\Phi = BV_0/I_a$, where V_0 is the volume of the irradiated solution, 3×10^{-3} L.

Measurement of Steady-State Fluorescence Emission: Fluorescence emission spectra were measured at room temperature with a Perkin-Elmer Instruments LS55 Luminescence Spectrometer. To determine the degree of fluorescence quenching of **1** and **2**, the fluorescence intensities of **1** and **2** were compared with the fluorescence intensity of tryptophan **5**, that is, $Q = 1 - (F_{TrpH-D}/F_{TrpH})$. Equal concentrations of tryptophan residue of the tryptophan-dimer and the free tryptophan **5** were used in the same solvent. An excitation wavelength of 290 nm was used, which corresponds to an absorption maximum of the model compounds resulting from an $n \rightarrow \pi^*$ transition of the indole unit.

pH-Dependent Measurement of the Quantum Yield for Splitting: To explore the effect of pH on the splitting reaction, Φ values were determined in solutions at various pH; water/tetrahydrofuran (80:20) solvent mixtures were used with 0.1 M buffer, over a pH range of 1 to 13. HCl/KCl buffers were used for pH 1–2, 0.1 M HCl/PAP (PAP = potassium hydrogen phthalate) buffers for pH 3, 0.1 M NaOH/PAP buffers for pH 4–5, potassium phosphate buffers were used for pH 6–11 and NaOH/KCl buffers were used for pH 12–13.

Tryptophan Methyl Ester (5):[28] Thionyl chloride (12 g, 100 mmol) was added dropwise to methanol (50 mL) at –5 °C whilst stirring. The mixture was stirred for a further 0.5 h at –5 °C. Tryptophan (10.0 g, 49 mmol) was added to the reaction solution. The ice bath was removed and the reaction mixture was allowed to stir for 5 h. The solution was heated to reflux and then concentrated in vacuo. The precipitate was recrystallized from ethanol/diethyl ether. The tryptophan methyl ester hydrochloride was dissolved in water, saturated sodium hydrogen carbonate solution was added to give pH 7–8 and the product **5** was extracted with ethyl acetate. The organic phase was washed with water, dried with $MgSO_4$, filtered and concentrated in vacuo to obtain **5** as a white powder (9.8 g, 92%). M.p. 90–91 °C. IR (KBr): $\tilde{\nu}$ = 3359(m), 3295 (w), 1732 (s), 1572 (m), 1227 (m), 742 (m) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.17 (m, 2 H, CH_2), 3.72 (s, 3 H, CH_3), 3.84 (m, 1 H, CH), 7.07 (s, 1 H, CH), 7.37 (m, 4 H, H_{indole}), 8.16 ppm (s, 1 H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 30.7 (CH_2), 52.0 (CH_3), 54.8 (CH), 110.3, 111.3, 118.5, 119.2, 121.9, 123.2, 127.3, 136.3, 175.7 ppm. TOF-MS (CI): $C_{12}H_{14}N_2O_2$ [M + 1]$^+$: 219.1134; found 219.1125.

Ethyl 1,2,3,4-Tetrahydro-5-methyl-2,4-dioxopyrimidine-1-propionate (6): Thymine (10.0 g, 79.3 mmol) was refluxed with ethyl acrylate (10.6 g, 106 mmol) in the presence of hydroquinone (100 mg, polymerization inhibitor) in ethanol (70 mL) containing NaOH (276 mg) for 20 h. After evaporation of the solvent, the residual material was washed several times with cold ethanol and recrystallized from ethanol to yield **6** as colorless needles (7.6 g, 42%). M.p. 153–154 °C. IR (KBr): $\tilde{\nu}$ = 3039 (m), 2994 (m), 1690 (s), 1464 (m), 1201 (m), 1013 (m), 797 (m) cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.25 (t, J = 7.0 Hz, 3 H, CH_2CH_3), 1.91 (s, 3 H, CH_3), 2.77 (d, J = 5.7 Hz, 2 H, CH_2), 3.97 (d, J = 5.7 Hz, 2 H, NCH_2), 4.15 (q, J = 7.0 Hz, 2 H, CH_2CH_3), 7.19 (s, 1 H, CH), 8.76 ppm (s, 1 H, NH). ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 11.9 (CH_3), 14.0 (CH_2CH_3), 32.9 (CH_2), 43.8 (CH_2), 60.2 (CH_2CH_3), 108.2 (C), 141.8 (CH), 150.8, 164.3, 170.7 ppm. TOF-MS (CI): $C_{10}H_{14}N_2O_4$ [M + 1]$^+$: 227.1032; found 227.1025.

Ethyl *cis*-[4*a*]-*cisoid*-[4*a*,4*b*]-*cis*-[4*b*]-Dodecahydro-4a,4b-dimethyl-2,4,5,7-tetraoxocyclobuta[1,2-*d*:4,3-*d*]dipyrimidine-1,8-dipropionate (7): The ethyl ester **6** (2.00 g, 8.8 mmol) was dissolved in acetone/MeCN (200 mL, 1:4) and the solution was degassed for 15 min in an ultrasonic bath. The solution was irradiated for 20 h with a 300-W high-pressure Hg lamp in a Pyrex photochemical reactor. During the irradiation the solution was purged with nitrogen. The reaction mixture was evaporated to dryness in vacuo. The residual oil was dissolved in EtOAc and subjected to column chromatography (silica gel-*H*, petroleum ether/EtOAc/MeOH, 3:2:0.1) to yield the isomers **7**. Recrystallization from petroleum ether/EtOAc yielded the *cis-syn* isomer **7** as colorless needles (720 mg, 36%). M.p. 157–158 °C. IR (KBr): $\tilde{\nu}$ = 3221 (m), 2985 (m), 1722 (s), 1679 (s), 1486 (m), 1211 (m), 1019 (m), 790 (m) cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.18 (m, 6 H, CH_2CH_3), 1.30 (s, 6 H, CH_3), 2.59 (m, 4 H, CH_2), 3.01 (m, 2 H, NCH_2), 3.76 (m, 2 H, NCH_2), 4.05 (m, 6 H, OCH_2 + CH), 10.31 ppm (s, 2 H, NH). ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 14.0 (CH_2CH_3), 18.0 (CH_3), 31.6 (CH_2), 41.8 (CH_2), 46.3 (C), 58.6 (CH), 60.1 (CH_2CH_3), 151.7, 170.3, 171.1 ppm. $C_{20}H_{28}N_4O_8$ (452.46): calcd. C 53.09, H 6.24, N 12.38; found C 53.24, H 6.01, N 12.17. TOF-MS (CI): $C_{20}H_{28}N_4O_8$ [M + 1]$^+$: 453.1985; found 453.1987.

***cis*-[4*a*]-*cisoid*-[4*a*,4*b*]-*cis*-[4*b*]-Dodecahydro-4a,4b-dimethyl-2,4,5,7-tetraoxocyclobuta[1,2-*d*:4,3-*d*]dipyrimidine-1,8-dipropionic Diacid (11):** The diester **7** (540 mg, 1.19 mmol) was dissolved in 5 M hydrochloride (12 mL). The reaction mixture was stirred refluxed for 1 h and then the reaction solution was concentrated in vacuo. The product was washed several times with Et_2O and dried in vacuo to yield a white powder (465 mg, 98%). M.p. 246–248 °C. IR (KBr): $\tilde{\nu}$ = 3214 (m), 3098 (m), 2920 (m), 1716 (s), 1485 (m), 1290 (m), 1213 (m) cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.29 (s, 6 H, CH_3), 2.53 (m, 4 H, CH_2), 2.99 (m, 2 H, NCH_2), 3.73 (m, 2 H, NCH_2), 4.05 (s, 2 H, CH), 10.31 (s, 2 H, NH), 12.36 ppm (s, 2 H, OH). ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 18.1 (CH_3), 31.7 (CH_2), 42.0 (CH_2), 46.4 (C), 58.9 (CH), 151.8, 170.5, 172.9 .

Model Compound 1: A solution of the *cis-syn*-thymine dimeric dicarboxylic acid **11** (200 mg, 0.51 mmol) and an excess of BOP (600 mg, 1.29 mmol) were dissolved in DMF (6 mL) and the mixture was stirred at room temperature for 30 min. After the addition of a solution of tryptophan methyl ester **5** (120 mg, 0.55 mmol) in DMF (2 mL), the reaction was stirred for 2 h at room temperature. Then pentylamine (0.12 mL, 1.04 mmol) was added to the reaction mixture, and stirring was continued for another 5 h at room temperature. The reaction mixture was diluted with water (100 mL) and extracted with $CHCl_3$ (3 × 200 mL). The combined organic layers were separated, dried with $MgSO_4$, filtered and concentrated in vacuo. The remaining crude product was purified by column chromatography (silica gel-*H*, $CHCl_3$/MeOH, 50:1). Compound **1** was obtained as a white powder (69 mg, 21%). M.p. 183–185 °C. IR (KBr): $\tilde{\nu}$ = 3348 (m), 3078 (m), 2953 (m), 2932 (m), 2864 (w), 1713 (s), 1650 (s), 1489 (m), 1289 (m), 1216 (m), 1108 (w), 745 (w) cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 0.85 (t, 3 H, CH_2CH_3), 1.20 (s, 3 H, CH_3), 1.23 (s, 3 H, CH_3), 1.30 (m, 6 H,

*(CH$_2$)$_3$*CH$_3$), 2.48 (m, 4 H, CH$_2$), 3.05 (m, 6 H, NCH$_2$ + NHCH$_2$ + CH*CH$_2$*), 3.57 (s, 3 H, OCH$_3$), 3.75 (m, 2 H, NCH$_2$), 3.89 (m, 2 H, CH), 4.48 (m, 1 H, *CH*CH$_2$), 7.24 (m, 5 H, H$_{indole}$), 7.94 (m, 1 H, CH$_2$*NH*), 8.47 (d, 1 H, CH*NH*), 10.27 (d, 2 H, NH), 10.86 ppm (d, 1 H, NH$_{indole}$). ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 13.8 (CH$_2$*CH$_3$*), 17.9 (CH$_3$), 18.1 (CH$_3$), 21.8 (CH$_2$), 27.0 (CH*CH$_2$*), 28.6 (CH$_2$), 28.7 (CH$_2$), 33.0 (CH$_2$), 33.4 (CH$_2$), 38.5 (CH$_2$), 42.4 (CH$_2$), 42.7 (CH$_2$), 46.0 (C), 46.7 (C), 51.8 (OCH$_3$), 53.3 (*CH*CH$_2$), 58.8 (CH), 59.0 (CH), 109.5, 111.5, 118.0, 118.5, 121.0, 123.6, 127.0, 136.1, 151.5, 170.0, 170.1, 170.2, 170.4, 170.5, 172.4 ppm. $C_{33}H_{43}N_7O_8$ (665.74): calcd. C 59.54, H 6.51, N 14.73; found C 59.59, H 5.94, N 14.45. ESI-MS (*m*/*z*): 666 [M]$^+$, 688 [M – H + Na]$^+$.

Model Compound 2: A solution of the *cis*-*syn*-thymine dimer dicarboxylic acid **11** (100 mg, 0.25 mmol) and an excess of BOP (300 mg, 0.65 mmol) were dissolved in DMF (4 mL) and the mixture was stirred at room temperature for 30 min. After the addition of a solution of tryptophan methyl ester **5** (120 mg, 0.55 mmol) in DMF (2 mL), the reaction was stirred for 5 h at room temperature. The reaction mixture was diluted with water (100 mL) and extracted with $CHCl_3$ (3 × 200 mL). The combined organic layers were separated, dried with $MgSO_4$, filtered and concentrated in vacuo. The remaining crude product was purified by column chromatography (silica gel-*H*, $CHCl_3$/MeOH, 50:1→20:1). Compound **2** was obtained as a white powder (51 mg, 25 %). M.p. 203–205 °C. IR (KBr): $\tilde{\nu}$ = 3372 (m), 3062 (m), 2954 (w), 1742 (s), 1710 (s), 1655 (s), 1487 (m), 1284 (m), 1204 (m), 1103 (m), 740 (m) cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.12 (s, 3 H, CH$_3$), 1.19 (s, 3 H, CH$_3$), 2.38 (m, 4 H, CH$_2$), 3.04 (m, 6 H, NCH$_2$ + CH*CH$_2$*), 3.55 (s, 3 H, OCH$_3$), 3.58 (s, 3 H, OCH$_3$), 3.72 (m, 2 H, NCH$_2$), 3.88 (m, 2 H, CH), 4.49 (m, 2 H, *CH*CH$_2$), 7.23 (m, 10 H, H$_{indole}$), 8.47 (m, 2 H, CH*NH*), 10.24 (s, 1 H, NH), 10.25 (s, 1 H, NH), 10.81 (s, 1 H, NH$_{indole}$), 10.84 ppm (s, 1 H, NH$_{indole}$). ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 17.9 (CH$_3$), 27.0 (CH*CH$_2$*), 32.9 (CH$_2$), 42.4 (CH$_2$), 45–46 (C), 51.9 (OCH$_3$), 53.3 (*CH*CH$_2$), 58.7 (CH), 109.4, 111.5, 118.0, 118.5, 121.1, 123.6, 127.0, 136.1, 151.5, 170.2, 170.5, 172.5 ppm. $C_{40}H_{44}N_8O_{10}$ (796.84): C 60.29, H 5.57, N 14.06; found C 59.21, H 5.34, N 13.55. ESI-MS (*m*/*z*): 797 [M]$^+$, 819 [M – H + Na]$^+$.

1-(Carboxyethyl)thymine Tryptophan Amide (3): The ethyl ester **6** (230 mg, 1.02 mmol) was dissolved in 5 M hydrochloride (10 mL) and the reaction mixture was refluxed for 1 h. The reaction solution was then concentrated in vacuo. The residue and an excess of BOP (600 mg, 1.29 mmol) were dissolved in DMF (6 mL) and stirred at room temperature for 30 min. After the addition of a solution of tryptophan methyl ester **5** (240 mg, 1.10 mmol) in DMF (2 mL), the reaction was stirred for 5 h at room temperature. The reaction mixture was purified by column chromatography on aluminium oxide (100–200 mesh, EtOAc/MeOH, 20:1→2:1). Compound **3** was obtained as a white powder (155 mg, 38 %). M.p. 110–112 °C. IR (KBr): $\tilde{\nu}$ = 3399 (m), 3057 (m), 2954 (m), 1738 (s), 1671 (s), 1460 (m), 1220 (m), 1127 (m), 1010 (m), 746 (m) cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.69 (s, 3 H, CH$_3$), 2.46 (t, *J* = 6.4 Hz, 2 H, CH$_2$), 3.06 (m, 2 H, CH*CH$_2$*), 3.55 (s, 3 H, OCH$_3$), 3.77 (t, *J* = 6.6 Hz, 2 H, NCH$_2$), 4.49 (m, 1 H, *CH*CH$_2$), 7.23 (m, 6 H, CH + H$_{indole}$), 8.47 (d, 1 H, CH*NH*), 10.83 (s, 1 H, NH$_{indole}$), 11.19 ppm (s, 1 H, NH). ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 11.9 (CH$_3$), 27.1 (CH*CH$_2$*), 34.0 (CH$_2$), 44.3 (CH$_2$), 51.8 (OCH$_3$), 53.2 (*CH*CH$_2$), 108.0 (C), 109.4, 111.5, 118.0, 118.5, 121.0, 123.6, 127.0, 136.0, 141.9 (CH), 150.7, 164.3, 169.8, 172.3 ppm. TOF-MS (CI): $C_{20}H_{22}N_4O_5$: [M + 1]$^+$: 399.1668; found 399.1667.

1-(Carboxyethyl)thymine *n*-Pentyl Amide (4): Compound **4** was synthesized similarly to compound **3** but with *n*-benzylamine instead of the tryptophan methyl ester **5**. Compound **4** was obtained as a white powder (185 mg, 68 %). M.p. 183–184 °C. IR (KBr): $\tilde{\nu}$ = 3362 (m), 2958 (m), 2929 (m), 1703 (s), 1671 (s), 1651 (s), 1547 (m), 1471 (m), 1361 (m), 1218 (m) cm^{-1}. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 0.82 (t, *J* = 6.8 Hz, 3 H, CH$_2$*CH$_3$*), 1.26 (m, 6 H, *(CH$_2$)$_3$*CH$_3$), 1.71 (s, 3 H, CH$_3$), 2.41 (t, *J* = 6.4 Hz, 2 H, CH$_2$), 2.99 (m, 2 H, NH*CH$_2$*), 3.81 (t, *J* = 6.4 Hz, 2 H, NCH$_2$), 7.36 (s, 1 H, CH), 7.91 (s, 1 H, *NH*CH$_2$), 11.19 ppm (s, 1 H, NH). ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 11.9 (CH$_3$), 13.8 (CH$_2$*CH$_3$*), 21.8 (CH$_2$), 28.5 (CH$_2$), 28.7 (CH$_2$), 34.4 (CH$_2$), 38.4 (CH$_2$), 44.7 (CH$_2$), 107.9 (C), 141.9 (CH), 150.7, 164.3, 169.3 ppm. TOFMS (CI): $C_{13}H_{21}N_3O_3$ [M + 1]$^+$: 268.1661; found 268.1664.

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