

# Do Photolyases Need To Provide Considerable Activation Energy for the Splitting of Cyclobutane Pyrimidine Dimer Radical Anions?

Qin-Hua Song,\* Wen-Jian Tang, Xue-Bao Ji, Hong-Bo Wang, and Qing-Xiang Guo<sup>[a]</sup>

**Abstract:** *cis-syn* Cyclobutane pyrimidine dimers, major UV-induced DNA lesions, are efficiently repaired by DNA photolyases. The key step of the repair reaction is a light-driven electron transfer from the FADH<sup>−</sup> cofactor to the dimer; the resulting radical anion splits spontaneously. Whether the splitting reaction requires considerable activation energy is still under dispute. Recent reports show that the splitting reaction of a dimer radical anion has a significant activation barrier (0.45 eV), and so photolyases have to provide considerable energy. However, these results contradict observations that *cis-syn* dimer radical anions split into monomers at −196°C, and

that the full process of DNA photo-reactivation was fast (1.5–2 ns). To investigate the activation energies of dimer radical anions, three model compounds **1–3** were prepared. These include a covalently linked cyclobutane thymine dimer and a tryptophan residue (**1**) or a flavin unit (**3**), and the covalently linked uracil dimer and tryptophan (**2**). Their properties of photosensitized splitting of the dimer units by tryptophan or flavin unit were investigated over a large temperature range,

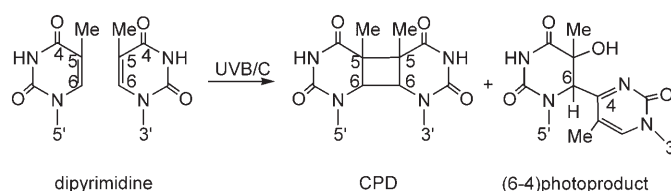
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−196 to 70°C. The activation energies were obtained from the temperature dependency of splitting reactions for **1** and **2**, 1.9 kJ mol<sup>−1</sup> and 0.9 kJ mol<sup>−1</sup> for the thymine and uracil dimer radical anions, respectively. These values are much lower than that obtained for *E. coli* photolyase (0.45 eV), and are surmountable at −196°C. The activation energies provide support for previous observations that repair efficiencies for uracil dimers are higher than thymine dimers, both in enzymatic and model systems. The mechanisms of highly efficient enzymatic DNA repair are discussed.

## Introduction

The far UV component of solar light (200–300 nm) has mutagenic, carcinogenic, and lethal effects on living organisms.<sup>[1]</sup> With the depleting of the atmospheric ozone layer, these effects will have increasingly serious consequences for the biosphere.<sup>[2]</sup> DNA is the most significant cellular target for far-UV light. The two major DNA lesions are the cyclobutane pyrimidine dimers (CPDs), which are in *cis-syn* form for double-stranded DNA (additionally *trans-syn* forms to a much lesser extent for single-stranded DNA), and pyrimi-

dine (6–4)pyrimidone photoproducts (Scheme 1). These instigate the harmful effects of UV, and constitute 70–80 %



Scheme 1. Two major UV-induced DNA photoproducts. Only photoproducts arising from adjacent thymines are shown.

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and 20–30 % of total photoproducts, respectively.<sup>[1,3]</sup> The two photolesions can be repaired through DNA photoreactivation, catalysed by CPD photolyases with high efficiencies ( $\Phi = 0.7–0.98$ ) and (6–4)photolyases with low efficiencies ( $\Phi = 0.05–0.10$ ) by utilizing the energy in sunlight (300–500 nm).<sup>[4]</sup>

CPD photolyases are monomeric proteins that contain two non-covalently bound chromophore cofactors. One

chromophore is a fully reduced flavin adenine dinucleotide ( $\text{FADH}^-$ ), the catalytic cofactor that carries out the repair upon excitation either by direct photon absorption or by resonance energy transfer from another chromophore (an antenna cofactor, methenyltetrahydrofolate or deazaflavin) that harvests sunlight. The model for the catalytic reaction is: 1) the enzyme binds CPD in a light-independent reaction, 2) the excited  $\text{FADH}^-$  transfers an electron to CPD to generate a charge-shifted radical pair ( $\text{FADH}^{\bullet-}\cdots\text{CPD}^{\bullet-}$ ), 3) the dimer radical anion ( $\text{CPD}^{\bullet-}$ ) undergoes spontaneous splitting and 4) back electron transfer restores the dipyrimidine and the functional form of the flavin, which is then ready for a new catalytic cycle.<sup>[4]</sup> In studies concerning DNA photorepair, chemists have been making tremendous efforts and have made significant contributions to an understanding of the mechanisms of CPD photolyase at the molecular level.<sup>[4b,5]</sup>

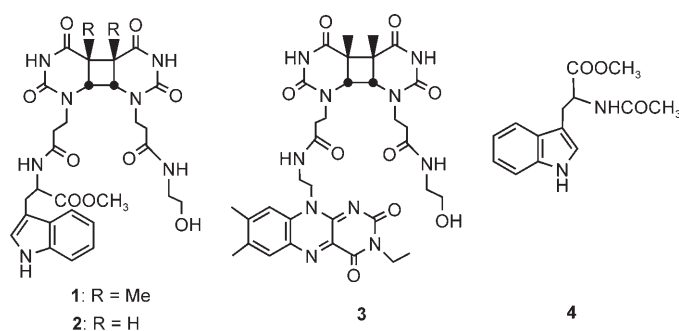
By utilizing model compounds, many fundamental questions concerning the details of the mechanism have been well resolved or rationalised,<sup>[6–10]</sup> such as the energetics of the enzymatic reaction, a non-synchronous concerted cleavage mechanism for C5–C5 and C6–C6 bonds, the electron transfer mechanism, the active form of the catalytic cofactor ( $\text{FADH}^-$ ), the distance requirement between the two cofactors and so on. Another goal for the synthesis of model compounds is the development of artificial DNA photolyases that are able to simulate the efficient recognition and repair of UV-induced DNA lesions.<sup>[11]</sup>

In spite of these efforts, however, there are still many unsolved questions concerning the details of the physical/chemical processes. Does the dimer radical anion require considerable additional activation energy ( $E_a$ ) for its splitting reaction to form dipyrimidine? What are the key factors in the highly efficient DNA repair by CPD photolyses? Early studies in a model system suggested that the splitting of the dimer radical anions had thermal requirements that could not be satisfied at  $-196^\circ\text{C}$ , and that the activation energy was reduced by CPD photolyase.<sup>[12]</sup> Furthermore, an ultrafast spectroscopic study of enzymatic DNA repair gave an activation barrier of 0.45 eV ( $\sim 43 \text{ kJ mol}^{-1}$ ) for the splitting reaction. This study examined the temperature dependency of the dimer splitting quantum yield in *E. coli* photolyase for dinucleotide substrates ( $\text{U} \leftrightarrow \text{T}$ ,  $\text{U} \leftrightarrow \text{U}$ ); no splitting was detected below  $-73^\circ\text{C}$ .<sup>[13]</sup> These findings suggested that the polypeptide chain provides some of the necessary activation energy for dimer splitting.<sup>[13,4c]</sup>

However, an activation energy of 0.45 eV contradicts observations that the *cis-syn* pyrimidine dimer anion split into monomers fast at  $-196^\circ\text{C}$ .<sup>[14]</sup> The activation energy for an intramolecular

reaction will give a much lower rate constant ( $10^5\text{--}10^7 \text{ s}^{-1}$ ) than the enzymatic splitting rate constant observed by ultrafast spectroscopy ( $> 10^9 \text{ s}^{-1}$ ).<sup>[15]</sup> In addition, the photolyase may have difficulty at such low temperatures ( $-183$  to  $-73^\circ\text{C}$ ), especially in a glassy state, for the active-site solvation that plays a critical role in DNA enzymatic repair.<sup>[16]</sup> Hence, there is a conflict over whether a considerable activation energy exists for the splitting reaction of dimer radical anions, and hence whether CPD photolyases need to provide additional energy to overcome the activation energy of the splitting.

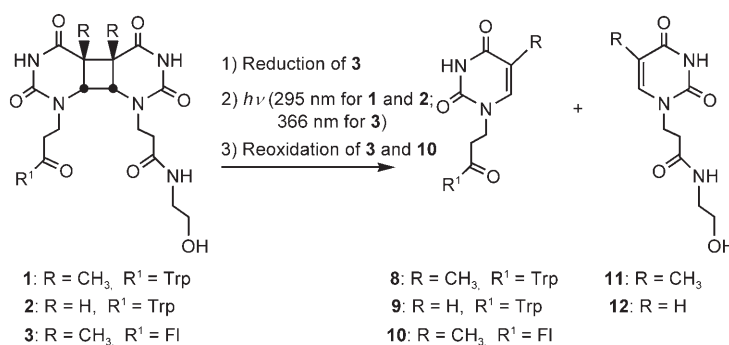
To answer this question three model compounds, **1–3**, were prepared. These contained a cyclobutane thymine or



uracil dimer covalently linked to a tryptophan (**4**) or a flavin moiety. The uracil dimer precursors **6** and **7** were prepared from **5**. Activation energies for the splitting of dimer radical anions were obtained from the temperature dependency of the cleavage of **1–3** into **8**<sup>[10a]</sup>–**12** (Scheme 2) over a wide temperature range ( $-196$  to  $60^\circ\text{C}$ ). Furthermore, new insights into the highly efficient repair mechanisms of CPD photolyases were gained.

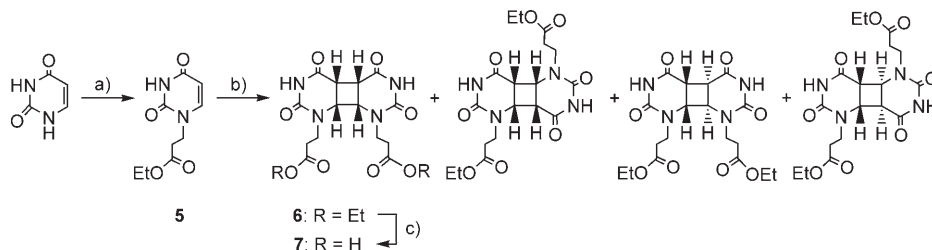
## Results and Discussion

**Synthesis of model compounds 1–3:** The *cis-syn* thymine dimer dicarboxylic acid was prepared from thymine,<sup>[10a]</sup> and the ethyl ester of 1-(2-carboxyethyl)uracil (**5**)<sup>[17]</sup> from uracil



Scheme 2. Cleavage reactions of model compounds **1–3** used to determine the activation energies of splitting dimer radical anions.

according to methods in the literature. The acetone/water solution of **5** was placed in a Pyrex photoreactor ( $\lambda > 290$  nm) accompanying to bubble with highly pure  $N_2$  and irradiated by a 300 W high-pressure Hg lamp. A mixture of all the four possible isomers was obtained (Scheme 3). The *cis-syn* uracil dimer (**6**) was separated by using a combina-



Scheme 3. a) Ethyl acrylate, hydroquinone, NaOH, EtOH, reflux 20 h; b)  $h\nu$  ( $> 290$  nm), acetone/ $H_2O$ , RT, 6 h; c) 5 M HCl, reflux 1 h.

tion of chromatography and selective precipitation.<sup>[18]</sup> The structure of the *cis-syn* isomer was inferred from the NOE spectrum and from comparison of the chemical shifts of four cyclobutane protons in their  $^1H$  NMR spectra with those from Carell et al.<sup>[18]</sup> for four benzyl-ester isomers of (carboxymethyl)-substituted uracil dimer, for which crystal structures of *cis-syn* and *trans-syn* isomers were reported. Subsequent hydrolytic cleavage of the ethyl ester gave the *cis-syn* uracil dimer dicarboxylic acid (**7**) as a white powder. This was further purified through recrystallisation in water. The crystal structure of **7** shows that it has a *cis-syn* isomer (Figure 1). This also confirmed the *cis-syn* structure of **6**.

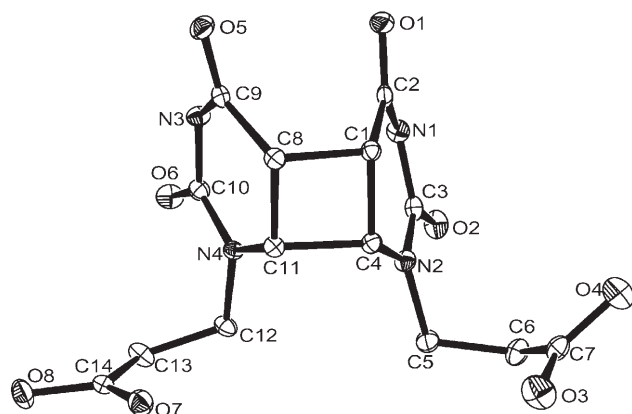
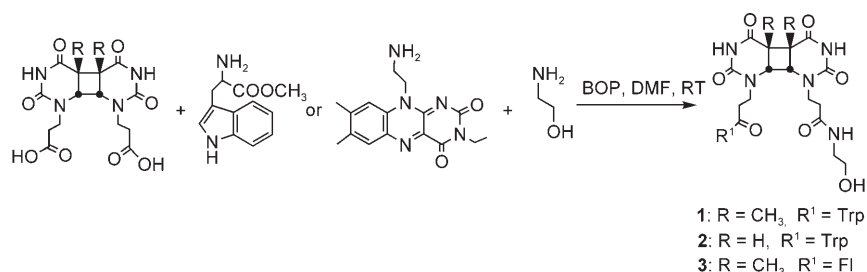


Figure 1. X-ray crystal structure of **7**. ORTEP plots of the molecular structures. Displacement ellipsoids are shown at the 50% probability level.

Compounds **1** and **2** were prepared by a condensation reaction between tryptophan methyl ester hydrochloride and either *cis-syn* thymine or uracil dimer dicarboxylic acid, with benzotriazol-1-yloxytris(dimethylamino)phosphonium-hexafluorophosphate (BOP) activation of the carboxylic acids in DMF (Scheme 4).<sup>[19]</sup> After adding one equivalent of the tryptophan methyl ester, the mixture was stirred for 2 h, then a large excess of 2-aminoethanol was added. This yielded **1** or **2** after 4–5 h. Compound **3** was synthesised in a similar fashion through the reaction of the *cis-syn* thymine dimer dicarboxylic acid with one equivalent of flavin,<sup>[9a]</sup> and the subsequent quenching of the reaction mixture with 2-aminoethanol.



Scheme 4.

**Photosplitting properties of model compounds 1–3:** All experiments were performed under identical solution conditions, a glycerol/water (1:1) solvent mixture with phosphate buffer. The sample solution (3 mL,  $\sim 5 \times 10^{-5}$  M) was prepared in a quartz cuvette (10 × 10 mm) with a rubber stopper. This was irradiated in a fluorescence spectrometer through a 10 nm slit with monochromatic light at 295 nm (for **1** and **2**) or 366 nm (for **3**). For preparation of the sample for **3** see the Experimental Section. Analysis of photolysis products by reverse-phase HPLC and co-injection of the expected synthesised photoproducts **8–12**<sup>[10a]</sup> confirmed that the model compounds split to give only **8** and **11** (for **1**), only **9** and **12** (for **2**) and only **10** and **11** (for **3**). Thus the splitting reactions were judged to be clean conversions as no other photoproducts were detected.

The photophysical/photochemical processes of **1–3** (represented as S–D) are detailed in Figure 2. Because the efficiency of the dimer splitting reactions is not concentration-dependent at 0.01–0.1 mM (within experimental error), the splitting reactions occur by an intramolecular electron transfer. Upon irradiation with light (295 nm for **1** and **2**, 366 nm for **3**), the tryptophan or flavin moiety absorbs a photon to produce the excited state ( $^1S^*D$ ). The excited state has the following relaxation pathways: fluorescence ( $k_f$ ), internal

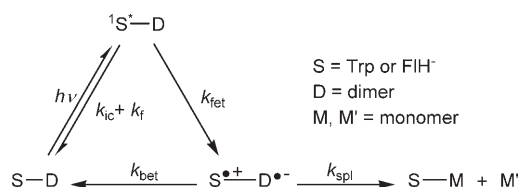


Figure 2. Photophysical and photochemical processes of model compounds under UV light irradiation.

conversion ( $k_{ic}$ ), and electron transfer to a covalently linked dimer ( $k_{fet}$ ). The charge-separated species ( $S^+-D^{\bullet-}$ ), formed by the electron transfer, undergoes two competitive processes: splitting ( $k_{spl}$ ) to produce  $M'$  and  $S^+-M'^{\bullet-}$  (this then becomes  $S-M$  by charge combination), and electron transfer ( $k_{bet}$ ) to return to the starting substrate.

**Temperature dependency of fluorescence for model compounds 1 and 2:** Quantum fluorescence yields for **1** and **2** were measured by using a 0.1 N NaOH aqueous solution of fluorescein as reference ( $\Phi_f = 0.87$ ).<sup>[20]</sup> With increasing temperature from  $-20$  to  $+60^\circ\text{C}$  quantum yields of fluorescence decrease, such as from 0.17 to 0.037 (**1**) and 0.16 to 0.036 (**2**) (Table 1). However, over this temperature range the extent

Table 1. Quantum yields of fluorescence and dimer splitting reaction, and extents of fluorescence quenching, for compounds **1**, **2** and **4**.<sup>[a]</sup>

$T [^\circ\text{C}]$	$\Phi_f$			$\varphi_{fet}$		$\Phi_{spl}$	
	<b>1</b>	<b>2</b>	<b>4</b>	<b>1</b>	<b>2</b>	<b>1</b>	<b>2</b>
$-196$	0.46	0.44	0.65	0.30	0.32	0.010	0.016
$-20$	0.17	0.16	0.47	0.64	0.66	0.125	0.180
$0$	0.12	0.11	0.33	0.64	0.67	0.121	0.187
$+20$	0.075	0.072	0.20	0.63	0.64	0.138	0.182
$+40$	0.050	0.048	0.14	0.64	0.66	0.143	0.184
$+60$	0.037	0.036	0.10	0.63	0.64	0.151	0.195

[a] Average of three measurements, error within  $\pm 5\%$ ; glycerol/water (1:1) solvent mixture with a buffer (50 mM phosphate, pH 7.5).

of fluorescence quenching,  $Q (=1-\Phi_f/\Phi_f^0)$ , in which  $\Phi_f$  is the quantum yield of fluorescence of **1** or **2**, and  $\Phi_f^0$  is that for free tryptophan (**4**) remains at approximately 0.64 for **1** and 0.66 for **2** (Table 1). The value of  $Q$  might reflect the quantum efficiency of the forward electron transfer  $k_{fet}$ , that is,  $Q = \varphi_{fet}$ .<sup>[10a]</sup> The results imply that, with increasing temperature, a decrease in fluorescence ( $k_f$ ) was replaced by an increase in internal conversion ( $k_{ic}$ ). At  $-196^\circ\text{C}$ , the quantum yields of fluorescence increase vastly for **1** ( $\Phi_f = 0.46$ ) and **2** ( $\Phi_f = 0.44$ ), while the values of  $\varphi_{fet}$  decrease (0.30 for **1** and 0.32 for **2**, Table 1) to 50% below those at other temperatures.

**Temperature dependence of splitting quantum yields of compounds 1–3.** The splitting quantum yields ( $\Phi_{spl}$ ) of compounds **1–3** are molecules of dimer split per photon absorbed. In the case of **1** and **2**, sample solutions ( $3\text{ mL}$ ,  $\sim 5 \times 10^{-5}\text{ M}$ ) were placed in quartz cuvettes with rubber stoppers, and irradiated at 295 nm in a fluorescence spectrometer.

The measurement of splitting quantum yield was described in our previous paper.<sup>[10a]</sup> The splitting quantum yields were obtained at various temperatures from  $-20$  to  $70^\circ\text{C}$ , and partial data are shown in Table 1. Using the measured  $\Phi_{spl}$  values at  $20^\circ\text{C}$  as references (0.138 for **1**, 0.182 for **2** and 0.048 for **3**),  $\Phi_{spl}$  values at  $-196^\circ\text{C}$  were obtained with a quartz tube by measuring changes in irradiation at  $-196^\circ\text{C}$  and by comparing with those at  $20^\circ\text{C}$  (Tables 1 and 2).

Table 2. Quantum yields of dimer splitting for compound **3**.<sup>[a]</sup>

$T [^\circ\text{C}]$	$\Phi_{spl}$	$T [^\circ\text{C}]$	$\Phi_{spl}$
$-196$	$< 0.005$	$+10$	0.079
$-20$	0.066	$+20$	0.048
$-10$	0.082	$+50$	0.052

[a] Average of two measurements; error within  $\pm 10\%$ ; glycerol/water (1:1) solvent containing 10 mM sodium dithionite with a buffer (50 mM phosphate, pH 8.0).

The data in Table 1 show that  $\Phi_{spl}$  is fairly constant over the range  $-20$  to  $+60^\circ\text{C}$ , but one order of magnitude lower at  $-196^\circ\text{C}$ . However, the quantum efficiency of forward electron transfer,  $\varphi_{fet}$ , over  $-20$  to  $+70^\circ\text{C}$ , is only twice that at  $-196^\circ\text{C}$ . This implies that the splitting of the dimer radical anion at  $-196^\circ\text{C}$  may be slowed, and/or back electron transfer may be enhanced, leading to more of a decrease of  $\Phi_{spl}$  than of  $\varphi_{fet}$ . This observation is similar to that seen with photolyase.<sup>[13]</sup>

The splitting quantum yields show that the uracil dimer unit in **2** ( $\Phi_{spl} = 0.18\text{--}0.20$ ) is split more efficiently than the thymine dimer unit in **1** ( $\Phi_{spl} = 0.13\text{--}0.15$ ). These results are in good agreement with ultrafast spectroscopy observations of DNA photolyase repair activity,<sup>[13]</sup> and steady-state studies.<sup>[9f]</sup> Carell and co-workers investigated the relative cleavage vulnerability of thymine and uracil cyclobutane dimer units in two model compounds that contained covalently connected flavin. In contrast to all previous findings,<sup>[21]</sup> cleavage data revealed faster repair of the sterically less encumbered uracil dimer than the thymine dimer. Stereoelectronic effects have been proposed as a probable explanation on the basis of ab initio calculations of the Mayer bond orders of the natural dimers and their radical anions.<sup>[9f]</sup> In this work, the difference in splitting quantum yields can be easily rationalised by the differences in the activation energy (vide infra) and efficiency of forward electron transfer ( $\sim 0.66$  for **2**,  $\sim 0.64$  for **1** between **2** and **1**).

The quantum yields of dimer splitting for **3** (flavin-containing) were measured at several temperatures by HPLC chromatography (see Experimental Section, Table 2). It is worth noting that a measurable quantum yield was obtained at  $-196^\circ\text{C}$ , which shows that the splitting reaction can occur at this temperature.  $\Phi_{spl}$  values are in the range 0.05 to 0.08 at  $-20$  to  $+50^\circ\text{C}$ , and reveal no significant temperature effect. This shows that the activation energy of the splitting reaction is not the determining factor for splitting efficiency. The finding further shows that the splitting reaction of the

dimer radical anion does not have a considerable activation barrier.

**Activation energies for the splitting reactions of dimer radical anions:** If a negligible temperature dependency of back electron transfer is assumed over  $-20$  to  $+70^\circ\text{C}$ ,  $\Phi_{\text{spl}}$  would reflect predominantly the temperature dependency of the splitting rate constant,  $k_{\text{spl}}(T)$  [Eq. (1)], in which  $\Phi_{\text{spl}} = \varphi_{\text{fet}} \varphi_{\text{spl}}$  and  $k_{\text{spl}}(T) = A_{\text{spl}} e^{-E_a/RT}$ , thus giving Equation (2).

$$\varphi_{\text{spl}} = k_{\text{spl}}(T) / (k_{\text{spl}}(T) + k_{\text{bet}}) \quad (1)$$

$$\ln(\varphi_{\text{fet}} / \Phi_{\text{spl}} - 1) = E_a / RT + \ln(k_{\text{bet}} / A_{\text{spl}}) \quad (2)$$

The values of  $\varphi_{\text{fet}}$  ( $Q$ ) and quantum yields  $\Phi_{\text{spl}}$  for compounds **1** and **2** were measured at various temperatures. Plots of  $\ln(\varphi_{\text{fet}} / \Phi_{\text{spl}} - 1)$  versus  $1/RT$  were made (Figure 3) by

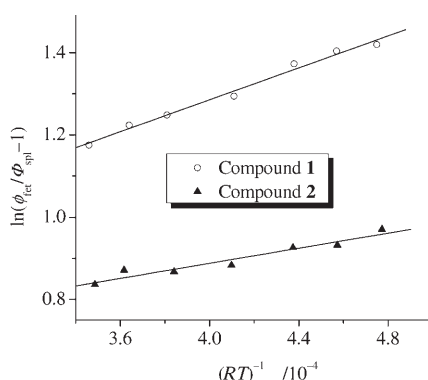


Figure 3. Plots of  $\ln(\varphi_{\text{fet}} / \Phi_{\text{spl}} - 1)$  versus  $1/RT$  for compounds **1** and **2**.

using Equation (2). Each activation energy  $E_a$  was obtained from the slope of linear regression ( $1.9 \pm 0.05 \text{ kJ mol}^{-1}$  for **1**, and  $0.9 \pm 0.06 \text{ kJ mol}^{-1}$  for **2**). The values of  $E_a$  are lower than the free energy of activation ( $1.3 \text{ kcal mol}^{-1}$ ,  $\Delta\Delta G^\ddagger = \Delta\Delta G_{\text{spl}}^\ddagger - \Delta\Delta G_{\text{bet}}^\ddagger$ ) as determined by Kim and Rose by using a covalently linked dimer-indole compound,<sup>[12b]</sup> in which the dimer was the cycloaddition product of orotic acid with thymine. This focused on solvent effects on the activation parameters ( $\Delta\Delta G^\ddagger$ ,  $\Delta\Delta H^\ddagger$ ,  $\Delta\Delta S^\ddagger$ ) in two different solvent systems.

Our results imply that splitting reactions for dimer radical anions have virtually no energy barrier. The results from quantum calculation<sup>[22]</sup> and ultrafast spectroscopy<sup>[23]</sup> showed that the energy barrier for the first bond scission of the dimer radical anion (C5–C5' or C6–C6') was low or zero. By using secondary deuterium isotope effects, Austin et al. studied the mechanism of dimer splitting both with photolyase and with model systems.<sup>[6]</sup> They concluded that fragmentation occurs by a non-synchronous concerted splitting reaction following electron addition to the dimer. Similarly, a study of the initial products of radiation-generated electrons to thymine and uracil dimers by EPR spectroscopy at

$-196^\circ\text{C}$  concluded that the monobonded intermediate is very unstable and breaks to give one monomer and one monomer radical anion.<sup>[5a]</sup> A more exact measurement showed that the entire process of the thymine dimer radical anion split and electron return was completed in 560 ps, by observation of the intermediate  $\text{FADH}^\cdot$  decay.<sup>[16]</sup> This shows that the full ring opening process of the dimer radical anion is very fast. Therefore, the second bond scission should also be a reaction with a low energy barrier, and thus the activation energy of the full splitting process would be low.

EPR observation of electron addition causing *cis-syn* pyrimidine dimers to split into monomers at  $-196^\circ\text{C}$  showed that the activation energy of the splitting reaction can be overcome at  $-196^\circ\text{C}$ .<sup>[14]</sup> In addition, it was confirmed that stereo repulsion between two pyrimidine rings for the *cis* isomer radical anion was stronger than that for the *trans* isomer, because *trans* isomer radical anions did not split at  $-196^\circ\text{C}$ .<sup>[14]</sup> Similar results were obtained from covalently linked dimer-flavin model systems.<sup>[9f]</sup> The splitting efficiencies of *cis-syn* dimer-flavin models were ten times that of *trans-syn* models, in a photosensitized electron transfer from fully reduced flavin to dimer at room temperature. The strain energy of *cis-syn* dimer favours its splitting reaction.

Besides a very low activation energy, the efficient *cis-syn* dimer splitting at  $-196^\circ\text{C}$  by electron addition is due to the absence of the competitive reaction with dimer splitting, back electron transfer, which occurs in the covalently linked models. In contrast to the electron addition to free dimers, the splitting of dimer radical anions has to compete with back electron transfer in covalently linked model systems,<sup>[7,10a,9,12]</sup> and enzyme systems.<sup>[13,24]</sup> At low temperatures, especially in a glassy state, back electron transfer cannot be efficiently suppressed and active-site solvation cannot be achieved.<sup>[16]</sup> In addition, the splitting rates for dimer radical anions are also slowed to some extent at low temperature. Hence, back electron transfer is dominant in the competition with the splitting reaction, and the repair reaction becomes trapped in equilibrium between forward and back electron transfer, without leading to dimer splitting.<sup>[13,24a]</sup>

**Mechanisms of the highly efficient enzymatic repair:** In a recent ultrafast spectroscopic study of dinucleotide thymine dimer repair by *E. coli* photolyase,<sup>[16]</sup> active-site solvation was observed on picosecond to nanosecond timescales, and was concluded to have a critical role in the continuous modulation of catalytic reactions. The active-site solvation in photolyase was thought to be critical to the strategic slowing down of the charge separation (170 ps) and recombination, by dynamically tuning the redox potentials of reaction species and stabilizing the charge-separated radical intermediates. This left enough time for cleavage of the cyclobutane ring (560 ps) and thus attained a maximum-repair quantum yield (0.87). For the covalently linked substrate-chromophore model systems, the biggest shortcoming is that they cannot mimic the characteristics of the noncovalent complex, which is formed by UV-damaged DNA and photolyase with a noncovalently bound flavin cofactor. In model sys-



tems with a modified  $\beta$ -cyclodextrin, we provided support that active-site solvation could suppress back electron transfer to give a high repair efficiency.<sup>[10c]</sup> The active-site solvation would not be achieved in rigid glass below  $-73^{\circ}\text{C}$ , and back electron transfer would not be suppressed. Hence, the fact that CPD photolyases retard back electron transfer by active-site solvation is one important factor for achieving highly efficient repair.

Back electron transfer leading to low efficiencies in model systems is widely accepted. However, we compared the quantum yields of tryptophan–thymine oxetane systems<sup>[10b]</sup> with those of flavin–oxetane,<sup>[9g]</sup> which were used as models for the (6–4)photolyase–substrate complex. We concluded that the main factor in model systems with low efficiencies was fast internal conversion  $k_{\text{ic}}$  of the excited flavin (for flavin model systems) or back electron transfer (for tryptophan model systems).<sup>[10b]</sup> This conclusion is supported by the CPD-containing model systems in this work, in which splitting efficiencies were 0.13–0.15 for the tryptophan–dimer system **1** and 0.05–0.08 for the flavin–dimer system **3**. This conclusion is based on the driving forces for back electron transfer in the two model systems. The driving forces of back electron transfer (charge recombination) for intermediates,  $\text{S}^{\bullet-}\text{--D}^{\bullet-}$ , can be estimated in the terms the Equation (3) in which  $E_{\text{ox}}$  and  $E_{\text{red}}$  are the redox potentials for chromophores and dimers, respectively.

$$-\Delta G_{\text{bet}} = E_{\text{ox}} - E_{\text{red}} - e^2/\epsilon a \quad (3)$$

For flavin–dimer systems, back electron transfer is a charge shift and no coulomb interaction occurs; the coulomb term for tryptophan systems can be neglected in aqueous solution. Because oxidation potentials are  $-0.48\text{ V}$  (versus a saturated calomel electrode (SCE)) for fully reduced flavin,<sup>[25]</sup> and  $0.86\text{ V}$  (versus SCE) for tryptophan,<sup>[26]</sup> the value of  $-\Delta G_{\text{bet}}$  for tryptophan systems is higher than that for the flavin–dimer system; the rate of back electron transfer in  $\text{Trp}^{\bullet+}\text{--D}^{\bullet-}$  would be faster than that in  $\text{FlH}^{\bullet+}\text{--D}^{\bullet-}$ . However, the higher efficiencies for tryptophan systems (with a faster back electron transfer) compared to flavin systems (with a slower back electron transfer) were unexpected. Hence, the main cause of low efficiency for flavin systems is not back electron transfer, but the internal conversion  $k_{\text{ic}}$ , which competes with forward electron transfer  $k_{\text{fet}}$  (Figure 2).

Another important factor for the high repair efficiency of CPD photolyase can be deduced from this: the enzyme could be efficiently suppressing internal conversion of the excited  $\text{FADH}^{\bullet-}$  by binding the cofactor. This deduction is also supported by reported experimental data: in the absence of a substrate, the lifetimes of the excited singlet state of  $\text{FADH}^{\bullet-}$  was  $1.8\text{ ns}$  in *E. coli* photolyase at  $2^{\circ}\text{C}$ ,<sup>[13]</sup> and  $1.5\text{ ns}$  in glucose oxidase.<sup>[27]</sup> The lifetime of the main fast-decaying component was  $17\text{ ps}$  compared the  $1.5\text{ ns}$  decay for free  $\text{FADH}^{\bullet-}$ , which gives a short lifetime. The complexity of the photophysics of free  $\text{FADH}^{\bullet-}$  in aqueous solution was considered to be related probably to the multiplicity of

structural configurations.<sup>[27]</sup> Easily changed conformations of free  $\text{FADH}^{\bullet-}$  would lead to a fast internal conversion, which would give a short lifetime. In the flavoproteins the flavin exists in more strictly defined conformations, and rapid structural modifications are suppressed because of constraints imposed by the proteic matrix, including various non-bond interactions at the active sites.<sup>[27]</sup> Hence, internal conversion is efficiently suppressed, which results in a long lifetime.

## Conclusion

This investigation has revealed that the splitting reaction of the dimer radical anion has a very low activation energy, and does not require considerable additional energy. Furthermore, more understanding of the highly efficient repair mechanism for CPD photolyase was gained.

The splitting quantum yields of three model compounds were determined over the temperature range  $-196$  to  $60^{\circ}\text{C}$ . From the temperature dependency of splitting quantum yields, very small activation energies were obtained,  $1.9$  and  $0.9\text{ kJ mol}^{-1}$  for thymine and uracil dimer radical anion, respectively. These results can well explain previous observations from various model systems and CPD photolyase systems. The energy barriers are not insurmountable at  $-196^{\circ}\text{C}$ , and so *cis-syn* dimer radical anions can split into monomers at  $-196^{\circ}\text{C}$ . For covalently linked chromophore–dimer model systems, the splitting reaction of the dimer radical anion does not easily occur at very low temperature, because there is a fast competitive reaction, back electron transfer, which is almost independent of temperature.

On the basis of the analysis and comparison of quantum yields and driving forces of charge recombination for their intermediates ( $\text{S}^{\bullet+}\text{--D}^{\bullet-}$ ) of **1** with **3**, we have concluded that internal conversion of the excited  $\text{FADH}^{\bullet-}$  in proteins is well suppressed. This leads to a highly efficient forward electron transfer and achieves highly efficient repair. In addition, data for **1** and **2** further showed a higher instability of the uracil dimer radical anion than for the thymine dimer radical anion.

## Experimental Section

**General methods:** All materials were obtained from commercial suppliers and used without further purification. Solvents of technical quality were distilled prior to use. Water was deionised and subsequently double distilled. The acetyl tryptophan methyl ester **4** was prepared from tryptophan methyl ester hydrochloride. Flavin was synthesised according to the method in the literature.<sup>[9a]</sup> Samples in all measurements were in a glycerol/water (1:1) solvent mixture with phosphate buffer ( $50\text{ mM}$ ,  $\text{pH } 7.5$  for **1** and **2**,  $\text{pH } 8.0$  for **3**).  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker AV spectrometer ( $300\text{ MHz}$  for  $^1\text{H}$ ,  $75\text{ MHz}$  for  $^{13}\text{C}$ ). The chemical shifts were referenced to acetone ( $\delta = 2.05, 29.8\text{ ppm}$ ) in  $[\text{D}_6]\text{acetone}$ , or  $\text{D}_2\text{O}$  and  $\text{DMSO}$  ( $\delta = 2.50, 39.5\text{ ppm}$ ) in  $[\text{D}_6]\text{DMSO}$  for  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra, respectively. Mass spectra were measured on a Bruker BIFLEX<sup>TM</sup> III Mass spectrometer. Elemental analysis was performed at the Analytic Center of University of Science and Technology of China.

FTIR spectra were recorded on a Bruker VECTOR22 spectrometer. UV/Vis spectra were measured on a Shimadzu UV-2401PC spectrometer. Fluorescence emission spectra were measured on a Perkin-Elmer LS55 luminescence spectrometer.

**Measurements of quantum yields of splitting reactions:** Measurement of the quantum yield for **1** and **2** was described in detail in our previous paper.<sup>[10a]</sup> The measurement method for **3** was described in the literature.<sup>[9a]</sup> The sample solution (3 mL,  $\sim 5 \times 10^{-5}$  M) was sealed in a quartz cuvette with a rubber stopper. To reduce the flavin chromophore, the solution was first bubbled with highly pure nitrogen for 20 min, and then 0.5 mL of a sodium dithionite glycerol/water solvent mixture (1:1, 0.06 M) with the same buffer was added through a syringe. The complete reduction of the sample was monitored by monitoring its UV/Vis absorption or fluorescence emission spectrum. The assay solution was irradiated with monochromatic light ( $\lambda = 366$  nm) in a Shimadzu RF-5301PC fluorescence spectrometer with a 10 nm slit. During irradiation experiments, ten 50  $\mu$ L samples were removed by syringe from the assay solution at certain time intervals. Small vials were filled with the samples and placed in dark in air. All samples were analysed by HPLC to quantify the amount of **3** and **10**. HPLC was performed on an Agilent 1100 (1 mL min<sup>-1</sup>, detection at 260 nm) with a C-18 reversed-phase column and a water/methanol solvent mixture (40:60 v/v) as eluent. The increase of photoproduct **10** and the consumption of starting material **3** were plotted against irradiation time and the data fitted by using a monoexponential growth curve. The splitting rate for **3** was obtained from the monoexponential growth equation. The intensity of the irradiating light  $I_0$  was determined by using ferrioxalate actinometry.<sup>[28]</sup> The rate of photon absorbed was calculated from Beer's law,  $I_a = I_0(1 - 10^{-A_{366}})$ , and by taking into account the absorption of sample at 366 nm ( $A_{366}$ ) before irradiation. These values allowed the calculation of quantum yield  $\Phi_{\text{spl}}$  (= (rate of dimer split)/(rate of photon absorbed)).

**Measurements of steady-state fluorescence emission:** To determine the extent of fluorescence quenching for **1** and **2**, fluorescence intensities were compared to that for the corresponding tryptophan without an attached dimer, **4** ( $Q = 1 - F/F_0$ ). In order to calculate the value of  $Q$ , the concentrations of tryptophan residues in model compounds, and the free tryptophan **4**, were normalised according to the absorbance at 295 nm, which was controlled below 0.05. The quantum fluorescence yields for **1** and **2** were measured at various temperatures by using a 0.1 N NaOH aqueous solution of fluorescein as reference ( $\Phi = 0.87$ ).<sup>[20]</sup>

**Ethyl-*cis*-[4a]-*cisoid*-[4a,4b]-*cis*-[4b]-dodecahydro-2,4,5,7-tetraoxocyclobuta [1,2-*d*:4,3-*d'*]-dipyrimidine-1,8-dipropionate (6):** Compound **5**<sup>[17]</sup> (2.00 g, 9.4 mmol) was dissolved in acetone/H<sub>2</sub>O (200 mL, 7:3) and the solution was degassed for 15 min in an ultrasonic bath. The solution was irradiated for 6 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 filtered, and then concentrated in vacuo. The residual oil was dissolved in dichloromethane and subjected to column chromatography (silica gel-*H*, CHCl<sub>3</sub>/MeOH 100:1) to yield the *cis-syn* isomer **6** as white solid (106 mg, 5.3 %). M.p. 192–194 °C; <sup>1</sup>H NMR (300 MHz, [D<sub>6</sub>]DMSO, TMS):  $\delta = 1.18$  (t, <sup>3</sup>*J*(H,H) = 7.1 Hz, 6H; CH<sub>2</sub>CH<sub>3</sub>), 2.62 (m, 2H; CH<sub>2</sub>), 3.09 (m, 2H; NCH<sub>2</sub>), 3.61 (dd, <sup>3</sup>*J*(H,H) = 3.7 Hz, <sup>3</sup>*J*(H,H) = 2.2 Hz, 2H; CH), 3.72 (m, 2H; NCH<sub>2</sub>), 4.06 (q, <sup>3</sup>*J*(H,H) = 7.1 Hz, 4H; CH<sub>2</sub>CH<sub>3</sub>), 4.26 (dd, <sup>3</sup>*J*(H,H) = 3.7 Hz, <sup>3</sup>*J*(H,H) = 2.2 Hz, 2H; NCH), 10.36 ppm (s, 2H; NH); <sup>13</sup>C NMR (75 MHz, [D<sub>6</sub>]DMSO):  $\delta = 14.0$  (CH<sub>2</sub>CH<sub>3</sub>), 31.7 (CH<sub>2</sub>), 38.6 (CH), 41.9 (NCH<sub>2</sub>), 54.3 (NCH), 60.2 (CH<sub>2</sub>CH<sub>3</sub>), 152.2 (NCON), 167.1 (COO), 171.2 ppm (COCH); ESI-MS: *m/z*: calcd for C<sub>18</sub>H<sub>24</sub>N<sub>4</sub>O<sub>8</sub>: 425.4 [*M*+H]<sup>+</sup>; found: 425.0.

***cis*-[4a]-*cisoid*-[4a,4b]-*cis*-[4b]-Dodecahydro-2,4,5,7-tetraoxocyclobuta [1,2-*d*:4,3-*d'*]-dipyrimidine-1,8-dipropionic acid (7):** The diester **6** (260 mg, 0.61 mmol) was dissolved in 5 M hydrochloride (10 mL). The reaction mixture was stirred at the reflux temperature for 1 h. The reaction solution was concentrated in vacuo. The residual product was washed several times with Et<sub>2</sub>O and dried in vacuo to yield **7** as a white powder (210 mg, 94 %). M.p. >250 °C; <sup>1</sup>H NMR (300 MHz, [D<sub>6</sub>]DMSO, TMS):  $\delta = 2.41$ –2.59 (m, 4H; CH<sub>2</sub>), 3.06 (m, 2H; NCH<sub>2</sub>), 3.61 (dd, <sup>3</sup>*J* = 3.8 Hz, <sup>3</sup>*J* = 2.0 Hz, 2H; CH), 3.69 (m, 2H; NCH<sub>2</sub>), 4.28 (dd, <sup>3</sup>*J* = 3.8 Hz, <sup>3</sup>*J* = 2.0 Hz, 2H;

NCH), 10.35 ppm (s, 2H; NH); <sup>13</sup>C NMR (75 MHz, [D<sub>6</sub>]DMSO):  $\delta = 31.7$  (CH<sub>2</sub>), 38.7 (CH), 42.2 (NCH<sub>2</sub>), 54.5 (NCH), 152.3 (NCON), 167.3 (COO), 172.9 ppm (COCH); MS (ESI): *m/z*: calcd for C<sub>14</sub>H<sub>16</sub>N<sub>4</sub>O<sub>8</sub>: 367.3 [*M*-H]<sup>-</sup>; found: 367.1.

**X-ray crystal structure data of 7:** Colourless block; C<sub>14</sub>H<sub>16</sub>N<sub>4</sub>O<sub>8</sub>, *M*<sub>r</sub> = 368.31; monoclinic, space group *C*1c1,  $\rho_{\text{calcd}} = 1.567$  g cm<sup>-3</sup>, *Z* = 4, *a* = 15.260(5), *b* = 14.656(4), *c* = 7.037(2) Å;  $\beta = 97.257(4)^\circ$ , *V* = 1561.3(8) Å<sup>3</sup>, *T* = -160(2) °C,  $\mu(\text{Mo-K}\alpha) = 0.131$  mm<sup>-1</sup>, 10086 reflections collected, 2022 unique (*R*<sub>int</sub> = 0.0412), *R*<sub>1</sub> = 0.0276, *wR*<sub>2</sub> = 0.0618 [*I* > 2 $\sigma$ (*I*)]. CCDC-632243 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.

**Synthesis of model compounds 1–3:** The model compounds **1** and **2** were prepared by the condensation of tryptophan methyl ester hydrochloride with *cis-syn* thymine dimer dicarboxylic acid,<sup>[10a]</sup> or uracil dimer dicarboxylic acid with benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP) activation of the carboxylic acids in DMF.<sup>[19]</sup> A solution of the *cis-syn* thymine or uracil dimer dicarboxylic acid (0.50 mmol), tryptophan methyl ester hydrochloride (130 mg, 0.50 mmol) and an excess of BOP (600 mg, 1.3 mmol) were dissolved in DMF (6 mL) and stirred at room temperature for 30 min. After the addition of triethylamine (0.3 mL), the reaction was stirred for 2 h at RT. Then 2-aminoethanol (0.06 mL, 1.0 mmol) was added to the reaction mixture, and stirring was continued for another 5 h at RT. The reaction mixture was placed on an Al<sub>2</sub>O<sub>3</sub> column (100–200 mesh, EtOAc/MeOH 1:1) and the eluted solution was concentrated in vacuo. The remaining crude product was purified by column chromatography (silica gel-*H*, CHCl<sub>3</sub>/MeOH 20:1 to 5:1). Compounds **1** and **2** were recrystallised from ethanol/H<sub>2</sub>O as a mixture of diastereomers and obtained as white powders in 15 and 9 % yields, respectively. Compound **3** was synthesised in a similar fashion through reaction of *cis-syn* thymine dimer dicarboxylic acid with one equivalent of flavin<sup>[9a]</sup> and subsequent quenching of the reaction mixture with 2-aminoethanol. It was obtained as a white powder in 17 % yield.

**Model compound 1:** *R*<sub>f</sub> = 0.29 (CHCl<sub>3</sub>/CH<sub>3</sub>OH 3:1); m.p. 145–147 °C; <sup>1</sup>H NMR (300 MHz, [D<sub>6</sub>]acetone, TMS):  $\delta = 1.20$ , 1.36 (2s, 3H; CH<sub>3</sub>), 1.42, 1.43 (2s, 3H; CH<sub>3</sub>), 2.49–2.76 (m, 4H; CH<sub>2</sub>CO), 3.14–3.40 (m, 6H; NCH<sub>2</sub>+NHCH<sub>2</sub>), 3.59–3.65 (m, 2H; CH<sub>2</sub>OH), 3.69, 3.70 (2s, 3H; OCH<sub>3</sub>), 3.96 (m, 2H; CH<sub>2</sub>CH), 4.17 (m, 1H; NCH), 4.04 (m, 1H; NCH), 4.71 (m, 1H; CH<sub>2</sub>CH), 7.07–7.57 ppm (m, 5H; H<sub>indole</sub>); <sup>13</sup>C NMR (75 MHz, [D<sub>6</sub>]acetone):  $\delta = 17.9$  (CH<sub>3</sub>), (18.1, 18.3) (CH<sub>3</sub>), 27.6 (CHCH<sub>2</sub>), (33.7, 33.8), (34.1, 34.2), 42.2, 43.8, (43.9, 44.0), (47.5, 47.6) (CCH<sub>3</sub>), (48.3, 48.5) (CCH<sub>3</sub>), 52.6 (OCH<sub>3</sub>), (54.1, 54.2), (60.2, 60.3) (CH), (60.4, 60.6) (CH+CH<sub>2</sub>OH), 109.9, 112.2, (118.5, 118.6), 119.4, (121.9, 122.0), (124.2, 124.3), (127.7, 127.8), 137.0, 152.8, 152.9, (171.6, 171.7), (171.8, 172.1), (172.4, 172.5), 172.9, (173.5, 173.6 ppm); IR (KBr):  $\tilde{\nu} = 3429$ , 1730, 1717, 1686, 1486, 1389, 1279, 1190 cm<sup>-1</sup>; MS (MALDI-TOF): *m/z*: calcd for C<sub>30</sub>H<sub>37</sub>N<sub>7</sub>O<sub>9</sub>: 640 [*M*]<sup>+</sup>; found: 640; elemental analysis calcd (%) for C<sub>30</sub>H<sub>37</sub>N<sub>7</sub>O<sub>9</sub>·H<sub>2</sub>O: C 54.79, H 5.98, N 14.91; found: C 54.94, H 6.00, N 14.78.

**Model compound 2:** *R*<sub>f</sub> = 0.31 (CHCl<sub>3</sub>/CH<sub>3</sub>OH 3:1); m.p. 155–158 °C; <sup>1</sup>H NMR (300 MHz, [D<sub>6</sub>]acetone, TMS):  $\delta = 2.44$ –2.70 (m, 4H; CH<sub>2</sub>CO), 3.14–3.38 (m, 6H; NCH<sub>2</sub>+NHCH<sub>2</sub>), 3.56–3.65 (m, 2H; CH<sub>2</sub>OH), 3.69 (s, 3H; OCH<sub>3</sub>), 3.73–3.81 (m, 2H; CH<sub>2</sub>CH), 3.87–3.97 (m, 2H; CH), 4.26–4.48 (m, 2H; CH), 4.75 (m, 1H; CH<sub>2</sub>CH), 7.05–7.58 ppm (m, 5H; H<sub>indole</sub>); <sup>13</sup>C NMR (75 MHz, [D<sub>6</sub>]acetone):  $\delta = (27.9, 28.0)$  (CHCH<sub>2</sub>), 34.2, 34.4, (39.7, 39.9) (CH), (40.0, 40.1) (CH), 42.5, 43.8, (44.2, 44.4), 52.8 (OCH<sub>3</sub>), 54.3 (2C), 55.7 (NCH), (55.8, 56.1) (NCH), 60.9 (CH<sub>2</sub>OH), 110.3 (2C), 112.4, 118.9, (119.6, 119.7), 122.2, (124.5, 124.7), (128.1, 128.2), 137.3, 153.3, 153.5, (168.5, 168.6), 172.7, 172.8, 173.1, (173.7, 173.8 ppm); MS (MALDI-TOF): *m/z*: calcd for C<sub>28</sub>H<sub>33</sub>N<sub>7</sub>O<sub>9</sub>: 612 [*M*]<sup>+</sup>; found: 612.

**Model compound 3:** *R*<sub>f</sub> = 0.33 (CHCl<sub>3</sub>/CH<sub>3</sub>OH 3:1); m.p. 206–208 °C; <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O, [D<sub>6</sub>]acetone):  $\delta = 1.19$  (t, <sup>3</sup>*J* = 7 Hz, 3H; CH<sub>2</sub>CH<sub>3</sub>), 1.27 (s, 3H; CH<sub>3</sub>), 1.35 (s, 3H; CH<sub>3</sub>), 2.14 (m, 2H; CH<sub>2</sub>CO), 2.31 (s, 3H; CH<sub>3</sub>), 2.46 (s, 3H; CH<sub>3</sub>), 2.49 (m, 2H; CH<sub>2</sub>CO), 2.93 (m, 1H), 3.25 (m, 3H), 3.57–4.03 (m, 10H), 4.66 (m, 2H), 7.59 (s, 1H; H<sub>A</sub>), 7.67 ppm (s, 1H; H<sub>A</sub>); <sup>13</sup>C NMR (75 MHz, D<sub>2</sub>O, [D<sub>6</sub>]acetone):  $\delta = 11.9$  (CH<sub>2</sub>CH<sub>3</sub>), 17.1 (CH<sub>3</sub>), 17.3 (CH<sub>3</sub>), 18.5 (CH<sub>3</sub>), 20.6 (CH<sub>3</sub>), 32.4, 33.3, 36.1, 37.4, 41.4, 42.6, 43.2, 44.1, 46.8 (CCH<sub>3</sub>), 48.2 (CCH<sub>3</sub>), 59.2 (CH),

59.3 (CH), 59.6 (CH<sub>2</sub>OH), 115.7, 130.3, 131.0, 133.4, 134.0, 138.9, 147.8, 150.2, 152.3, 152.5, 156.6, 160.1, 172.2, 172.7, 173.2 ppm (2C); IR (KBr):  $\tilde{\nu}$  = 3429 (s), 1702 (s), 1648 (s), 1584 (s), 1548 (s), 1484 (m), 1461 (m), 1447 (m), 1288 (m), 1231 (m), 848 cm<sup>-1</sup> (m); MS (MALDI-TOF):  $m/z$ : calcd for C<sub>34</sub>H<sub>42</sub>N<sub>10</sub>O<sub>6</sub>: 735 [M]<sup>+</sup>; found: 735; elemental analysis calcd (%) for C<sub>34</sub>H<sub>42</sub>N<sub>10</sub>O<sub>6</sub>·3H<sub>2</sub>O: C 51.77, H 6.13, N 17.76; found: C 51.46, H 6.05, N 17.31.

**Synthesis of 9–12:** Compounds **9–12** were prepared by the similar condensation of *cis-syn* thymine/uracil dimer dicarboxylic acid<sup>[10a]</sup> with tryptophan methyl ester hydrochloride (for **9** and **10**) or 2-aminoethanol (for **11** and **12**) with BOP activation of the carboxyl acids in DMF.<sup>[19]</sup> These reactants were dissolved in DMF, and stirred for 2–3 h at RT. The reaction mixtures were purified by column chromatography. The compounds were obtained as white powders in ca. 40% yield.

**1-(Carboxyethyl)uracil tryptophan amide (9):**  $R_f$  = 0.79 (CHCl<sub>3</sub>/CH<sub>3</sub>OH 5:1); m.p. 179–181 °C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, TMS):  $\delta$  = 2.57 (t, <sup>3</sup>J = 6 Hz, 2H; CH<sub>2</sub>CO), 3.25–3.30 (m, 2H), 3.72 (s, 3H; CH<sub>3</sub>O), 3.95 (m, 2H), 4.90 (dd, <sup>3</sup>J(H,H) = 5 Hz, <sup>3</sup>J(H,H) = 8 Hz, 1H; NHCH), 5.55 (d, <sup>3</sup>J(H,H) = 8 Hz, 1H; COCH), 6.17 (d, <sup>3</sup>J(H,H) = 8 Hz, 1H; CHN), 6.92 (s, 1H; H<sub>indol</sub>), 7.08–7.47 (m, 4H; H<sub>indol</sub>), 8.16 ppm (s, 1H; CH<sub>2</sub>CONH); <sup>13</sup>C NMR (75 MHz, [D<sub>6</sub>]DMSO):  $\delta$  = 27.6 (CH<sub>2</sub>), 34.4 (CH<sub>2</sub>CO), 45.3 (CH<sub>2</sub>NH), 52.5 (CH<sub>3</sub>), 53.7, 101.0 (CHCO), 109.9, 112.0, 118.5, 119.1, 121.7, 124.1, 127.5, 136.5, 146.7 (CHN), 151.3 (NHCON), 164.5 (CH<sub>2</sub>CO), 170.5 (COO), 172.8 ppm (NCO); IR (KBr):  $\tilde{\nu}$  = 3373 (s), 3274 (s), 1727 (s), 1679 (s), 1353 (m), 1228 (s), 826 (m), 737 cm<sup>-1</sup> (m); ESI-MS:  $m/z$ : calcd for C<sub>19</sub>H<sub>20</sub>N<sub>4</sub>O<sub>5</sub>: 383.4 [M–H]<sup>–</sup>; found: 383.0.

**1-(Carboxyethyl)thymine flavin amide (10):**  $R_f$  = 0.40 (CHCl<sub>3</sub>/CH<sub>3</sub>OH 3:1); <sup>1</sup>H NMR (300 MHz, [D<sub>6</sub>]DMSO, TMS):  $\delta$  = 1.16 (t, <sup>3</sup>J(H,H) = 6.9 Hz, 3H; CH<sub>2</sub>CH<sub>3</sub>), 1.71 (s, 3H; CH<sub>3</sub>-Thy), 2.31 (t, <sup>3</sup>J = 6.3 Hz, 2H), 2.41 (s, 3H; CH<sub>3</sub>), 2.51 (s, 3H; overlap with CH<sub>3</sub>-DMSO), 3.48 (m, 2H; CH<sub>2</sub>), 3.72 (t, <sup>3</sup>J(H,H) = 6.3 Hz, 2H), 3.93 (q, <sup>3</sup>J = 6.9 Hz, 2H; CH<sub>2</sub>CH<sub>3</sub>), 4.64 (m, 2H; CH<sub>2</sub>), 7.39 (s, 1H; CH-Thymine), 7.90 (s, 1H; H<sub>Ar</sub>), 7.92 (s, 1H; H<sub>Ar</sub>), 8.25 (m, 1H; NH), 11.22 ppm (s, 1H; NH); <sup>13</sup>C NMR (75 MHz, [D<sub>6</sub>]DMSO):  $\delta$  = 12.0 (CH<sub>3</sub>-Thymine), 13.0 (CH<sub>2</sub>CH<sub>3</sub>), 18.8 (CH<sub>3</sub>), 20.8 (CH<sub>3</sub>), 34.1 (CH<sub>2</sub>), 35.5 (CH<sub>2</sub>), 36.0 (CH<sub>2</sub>), 43.3 (CH<sub>2</sub>), 44.3 (CH<sub>2</sub>), 108.1, 116.1, 131.0, 131.2, 134.2, 136.0, 136.2, 141.9, 146.7, 149.1, 150.7, 154.7, 159.3, 164.3, 170.6 ppm; ESI-MS:  $m/z$ : calcd for C<sub>24</sub>H<sub>27</sub>N<sub>7</sub>O<sub>5</sub>: 516.5 [M+Na]<sup>+</sup>; found: 516.2.

**1-(Carboxyethyl)thymine N-hydroxyethyl amide (11):**  $R_f$  = 0.12 (CHCl<sub>3</sub>/CH<sub>3</sub>OH 3:2); <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O, [D<sub>6</sub>]acetone):  $\delta$  = 1.88 (s, 3H; CH<sub>3</sub>), 2.67 (t, 2H; <sup>3</sup>J(H,H) = 6 Hz, CH<sub>2</sub>CO), 3.31 (t, 2H; <sup>3</sup>J(H,H) = 5 Hz, NHCH<sub>2</sub>), 3.61 (t, 2H; <sup>3</sup>J(H,H) = 5 Hz, CH<sub>2</sub>OH), 4.04 (t, 2H; <sup>3</sup>J(H,H) = 6 Hz, NCH<sub>2</sub>), 7.44 ppm (s, 1H; CH); <sup>13</sup>C NMR (75 MHz, D<sub>2</sub>O, [D<sub>6</sub>]acetone):  $\delta$  = 11.5 (CH<sub>3</sub>), 35.0 (CH<sub>2</sub>CO), 41.7 (NCH<sub>2</sub>), 45.9 (NHCH<sub>2</sub>), 60.2 (CH<sub>2</sub>OH), 110.9 (CH<sub>3</sub>C), 143.5 (CHN), 152.3 (NHCON), 167.2 (CH<sub>2</sub>CO), 173.4 ppm (NCO); ESI-MS:  $m/z$ : calcd for C<sub>10</sub>H<sub>13</sub>N<sub>3</sub>O<sub>4</sub>: 240.2 [M–H]<sup>–</sup>; found: 240.2.

**1-(Carboxyethyl)uracil N-hydroxyethyl amide (12):**  $R_f$  = 0.34 (CHCl<sub>3</sub>/CH<sub>3</sub>OH 5:1); m.p. 176–178 °C; <sup>1</sup>H NMR (300 MHz, [D<sub>6</sub>]DMSO, TMS):  $\delta$  = 2.47 (t, <sup>3</sup>J(H,H) = 6.6 Hz, 2H; CH<sub>2</sub>CO), 3.11 (t, <sup>3</sup>J(H,H) = 6.0 Hz, 2H; NHCH<sub>2</sub>), 3.37 (t, <sup>3</sup>J(H,H) = 6.0 Hz, 2H; NCH<sub>2</sub>), 3.84 (t, <sup>3</sup>J(H,H) = 6.6 Hz, 2H; CH<sub>2</sub>OH), 5.51 (d, <sup>3</sup>J(H,H) = 8 Hz, 1H; COCH), 7.52 (d, <sup>3</sup>J(H,H) = 8 Hz, 1H; NCH), 7.99 ppm (s, 1H; NHCH<sub>2</sub>); <sup>13</sup>C NMR (75 MHz, [D<sub>6</sub>]DMSO):  $\delta$  = 34.4 (CH<sub>2</sub>CO), 41.6 (CH<sub>2</sub>N), 45.1 (CH<sub>2</sub>NH), 59.9 (CH<sub>2</sub>OH), 100.7 (CHCO), 146.5 (CHN), 151.0 (NHCON), 164.2 (CH<sub>2</sub>CO), 170.2 ppm (NCO); IR (KBr):  $\tilde{\nu}$  = 3374 (s), 3306 (s), 3085 (m), 1726 (m), 1663 (s), 1559 (m), 1413 (m), 1076 (m), 831 cm<sup>-1</sup> (m); ESI-MS:  $m/z$ : calcd for C<sub>9</sub>H<sub>13</sub>N<sub>3</sub>O<sub>4</sub>: 250.2 [M+Na]<sup>+</sup>; found: 250.1.

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