A Possible Chain Reaction in Photosensitized Splitting of Pyrimidine Dimers by a Protonated, Oxidized Flavin

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Pyrimidine dimer cycloreversion was photosensitized by protonated 2',3',4',5'-tetraacetylriboflavin (ac_4rfH^+), whereas the protonated flavin radical ($ac_4rfH_2^{\bullet+}$) failed to sensitize detectable dimer splitting. In acetonitrile containing 0.075% perchloric acid, the ac_4rf absorption bands at 375 nm and 445 nm are replaced by a new band at 390 nm due to ac_4rfH^+. Irradiation of ac_4rfH^+ in the presence of *cis,syn-* or *trans,syn*-1,3-dimethyluracil dimer resulted in efficient cycloreversion to 1,3-dimethyluracil, probably induced by electron abstraction from the dimer by ac_4rfH^+ to produce the dimer radical cation. The quantum yields of splitting were large (Φ_{spl} = 0.35 and 1.5 for cis,syn and trans,syn isomers, respectively, at [dimer] = 1.2 mM). Splitting quantum yields increased with dimer concentration in a quadratic fashion. These results are consistent with the occurrence of a chain reaction propagated by monomer$^{\bullet+}$ + dimer → monomer + dimer$^{\bullet+}$ in the case of the dimethyluracil dimers. The fluorescence of the protonated flavin (λ_{em} = 507 nm) was found to be quenched by the dimers. In the presence of small amounts of added water, however, both the fluorescence quenching and splitting were abolished. Two cis,syn dimers in which the N(1)- and N(1')-atoms were tethered via a trimethylene bridge exhibited quantum yields of approximately 0.02 at the above dimer concentration. A compound in which ac_4rf is covalently bound to a pyrimidine dimer was also found to undergo splitting in acidified acetonitrile (Φ = 0.03). These values are more than 1 order of magnitude greater than those reported for unprotonated flavins.

Introduction

Interest in oxidized and reduced flavins as photosensitizers of pyrimidine dimer splitting arises from the utilization of a reduced flavin and either an oxidized deazaflavin or a reduced folate by photolyases.[1] These DNA repair enzymes bind to dimer-containing DNA and split the dimer in a subsequent, light-dependent step.[2] Photoinduced electron transfer to produce a dimer radical cation or anion is implicated in model studies of dimer cycloreversion,[3] although the direction of electron transfer between enzyme-bound sensitizer and dimer is unknown.[4] We have shown by simple MO theory that cycloreversion of the dimer radical anion occurs even though the process is orbital symmetry forbidden because a nonsynchronous concerted or fully stepwise splitting pathway experiences a kinetic acceleration induced by the electron in the singly occupied molecular orbital (SOMO).[5] Similar considerations may apply to the dimer radical cation.

It is well-known that flavins can serve as photo-oxidizing agents. The excited flavin singlet ($^1Fl^*$) readily intersystem crosses (Φ_{isc} = 0.67)[6] to the triplet state (^3Fl):

$$Fl \xrightarrow{h\nu} {}^1Fl^* \xrightarrow{\Phi_{isc} = 0.67} {}^3Fl$$

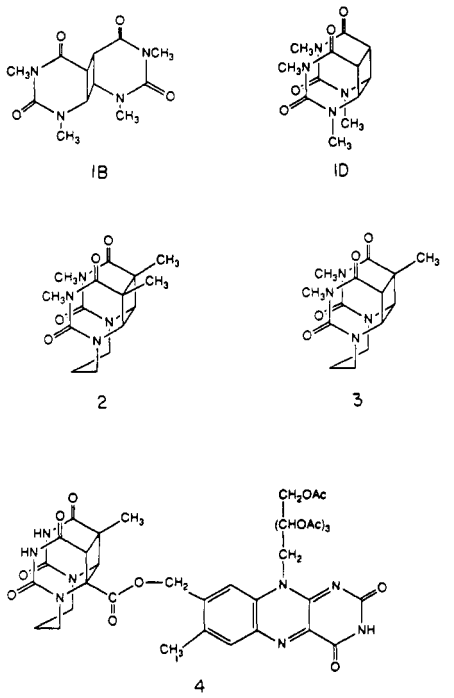
Electron abstraction from the substrate (SH_2) by the triplet flavin produces the oxidized substrate:

$$^3Fl \xrightarrow{SH_2} FlH_2 + S$$

Neutral excited flavins are relatively unreactive toward pyrimidine dimers ($\Phi_{spl} \simeq 10^{-3}$–$10^{-4}$),[7] possibly because of inefficient electron abstraction from the dimer by the flavin.[8] The protonated flavins have greater electron-abstracting capability,[9] but in aqueous solution, flavins are protonated only at very low pH ($pK_a \simeq 0$).[10] Protonation occurs more readily in acetonitrile.[9]

We now present[3i] the results of a study of a protonated flavin employed as a photosensitizer of pyrimidine dimer cycloreversion in acetonitrile. In addition to increased splitting efficiencies, we found evidence for a novel chain reaction in which dimer splitting was propagated by monomer radical cations.

Chart I



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Experimental Section

General. For dimer splitting experiments, light from an Oriel 500-W Hg-Xe deep-UV arc lamp was focused onto the entrance slit of a Jarrell-Ash 0.25-m monochromator. The emerging beam was then refocused onto a cuvette containing the sample. Samples were stirred during irradiation. NMR spectra were recorded at either 400 or 300 MHz. The apparatus used to measure fluorescence lifetimes by laser flash photolysis and single photon counting has been described.[11] The structures of compounds used in this study are shown in Chart I. (Dimers are given letter designations **B** and **D** consistent with the literature.[12]) Acetonitrile used for synthesis of compound 4 and for all photochemical measurements was dried by distillation from CaH_2. Thin-layer chromatography was carried out on silica gel plates with the following solvent systems: A, 3% methanol in ethyl acetate (by vol); B, ethyl acetate, 2-propanol, water (12,1,6, by vol; upper phase).

Synthesis of Compounds 1B, 1D, 2, 3, and ac_4rf. The isomeric 1,1′,3,3′-tetramethyluracil cyclobutadipyrimidines **1B** (trans,syn) and **1D** (cis, syn) were prepared and purified according to a literature procedure[12] and were recrystallized from ethanol. Syntheses of the *cis,syn*-3,3′-dimethyl-1,1′-trimethylenebis(thymine) cyclobutadipyrimidine **2** and the methylated, mixed dimer of thymine and uracil **3** are described in an earlier paper.[3b]

2′,3′,4′,5′-Tetraacetylriboflavin was prepared according to a literature procedure[13] and was recrystallized from 2-propanol.

Synthesis of Compound 4. 8α-Bromo-2′,3′,4′,5′-tetraacetylriboflavin[14] was coupled to the benzyltriethylammonium salt of the dimer carboxylic acid[15] in refluxing acetonitrile. After 1.5 h, the reaction was complete, as determined by the absence of the bromoflavin by TLC. The R_f values of the bromoflavin were 0.43 (system A) and 0.51 (system B), and the R_f values of **4** were 0.07 (system A) and 0.16 (system B). The product was purified by chromatography on 1000-μm preparative silica gel plates. High-resolution FAB MS (Midwest Center for Mass Spectrometry) of **4**: theoretical exact mass for $C_{38}H_{40}N_8O_{16} + H^+$ 865.264, found 865.262. Compound **4** was a mixture of two diastereomers, as evidenced by the presence of two sets of resonances for certain protons in the NMR spectrum recorded in acetone-d_6: NMR δ 1.52 [1 H, d, $-CH_2CHHCH_2-$], 1.64 and 1.66 [3 H, 2s, acetyl-CH_3], 1.74 and 1.76 [3 H, 2s, cyclobutyl-C(5)CH_3], 2.00 and 2.01 [3 H, 2s, acetyl-CH_3], 2.1 [m, $-CH_2CHHCH_2-$], 2.14 [3 H, s, acetyl-CH_3], 2.25 and 2.26 [3 H, 2s, acetyl-CH_3], 2.87 [3 H, s, flavin-C(7)CH_3], 3.66 and 3.67 [1 H, 2s, cyclobutyl-C(5)H], 4.25 [1 H, m, ribityl-C(5′)H_a], 4.30 [2 H, m, $-CHHCH_2CHH-$], 4.45 [1 H, m, ribityl-C(5′)H_b], 4.64 [1 H, cyclobutyl-C(6)H], 5.00 [1 H, m, ribityl-C(1′)H_a and H_b], 5.40 [1 H, m, ribityl-C(4′)H], 5.54 [1 H, m, ribityl-C(3′)H], 5.6 [2 H, m, C(8α)H_a and H_b], 7.97 [1 H, s, flavin-C(6 or 9)H] and 8.04 [1 H, d, J = 2.6 Hz, flavin-C(6 or 9)H], 9.39 [br s, NH], 9.52 and 9.57 [br s, NH], 10.3 [br s, flavin-N(3)H].

Scheme I

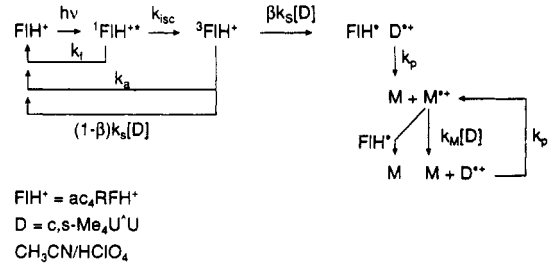

Table I. Quantum Yield of Splitting (Φ_{spl}) of Dimers 1B, 1D, 2, and 3

dimer[a]	Φ_{spl}	dimer[a]	Φ_{spl}
1B	1.5	**2**	0.016
1D	0.35	**3**	0.022

[a] In all cases [dimer] was 1.2×10^{-3} M. Solutions were aerobic and irradiations were carried out at 436 nm.

Dimer Splitting Experiments. Dimer splitting was typically carried out as follows. Acidified acetonitrile (90 μL of 70% perchloric acid per 100 mL of solution) was prepared in flame-dried glassware. Tetraacetylriboflavin was dissolved in acidified acetonitrile, and the absorbance of this solution was adjusted to 0.15 at the irradiation wavelength, thereby giving a flavin concentration of approximately 40 μM. A 0.5-mL aliquot of dimer dissolved in acetonitrile (without acid) was then added to 2.5 mL of the flavin solution in a 1.0-cm-path-length cuvet. Irradiation at 436 nm was carried out, and the solution was evaporated to dryness. The residue was dissolved in DMSO-d_6 for NMR spectroscopy. The extent of splitting was measured (at ~5% overall conversion) by following the increase in UV absorption due to the formation of the pyrimidine products over 60 s of irradiation. The λ_{max} of 1,3-dimethyluracil was shifted to 292 nm in acidified acetonitrile ($\epsilon_{292} = 8.7 \times 10^3$ M^{-1} cm^{-1}). The quantum yield was determined from a plot of ΔA_{292} vs time. For compound **4**, the ε of the *n*-butyl ester of 1-(3-thyminylpropyl)orotic acid was used to determine the extent of splitting (λ_{max} = 279 nm, $\epsilon_{279} = 1.4 \times 10^4$ M^{-1} cm^{-1} in acidified acetonitrile) from a plot of ΔA_{279} vs time (data taken at 20, 40, 60, and 80 s of irradiation). For quantum yield measurements of dimer **2**, ε of trimethylenebis(*N*(3)-methylthymine) was used ($\epsilon_{275} = 1.3 \times 10^4$ M^{-1} cm^{-1}). For dimer **3**, the corresponding split compound, 1-(*N*(3)-methylthymin-1-yl)-3-(*N*(3)-methyluracil-1-yl)propane was used (λ_{max} = 275 nm, $\epsilon_{275} = 1.3 \times 10^4$ M^{-1} cm^{-1} in acidified acetonitrile). Potassium ferrioxalate actinometry was used to measure the light output of the Hg–Xe lamp for quantum yield measurements; correction was required for incomplete absorption of the light by the actinometer solution at this wavelength. As in the past, the quantum yield of splitting refers to the number of dimers split per absorbed photon.

Results

In acidified acetonitrile, protonated 2′,3′,4′,5′-tetraacetylriboflavin (ac_4rfH^+) is formed, as revealed by the replacement of the typical oxidized flavin bands at 375 and 445 nm by a single new absorbance band (λ_{max} = 390 nm).

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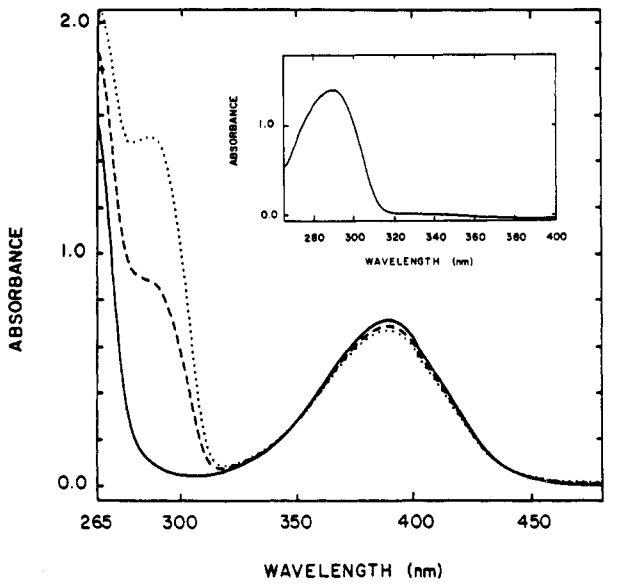

Figure 1. Spectrum of dimer **1D** and ac_4rfH^+ before (solid line) and after irradiation at 436 nm for 1 min (dashed line) and 2 min (dotted line). Inset: difference spectrum obtained after 2-min irradiation.

Irradiation of a solution of ac_4rfH^+ with dimer **1B** or dimer **1D** resulted in very efficient splitting to yield 1,3-dimethyluracil. Splitting was evidenced by a dramatic increase in UV absorption due to the formation of the pyrimidine 5,6-double bonds. The spectral changes that occur upon irradiation of a solution of **1D** and ac_4rfH^+ are shown in Figure 1. In the acidic medium, the λ_{max} of 1,3-dimethyluracil was shifted to 292 nm. The difference spectrum after 2 min of irradiation (Figure 1) had λ_{max} = 290 nm. Additionally, the identity of the product of the irradiation was confirmed to be 1,3-dimethyluracil by NMR spectroscopy after irradiation of solutions of **1B** and **1D**. The quantum yield for **1D** measured in argon-purged solution was found to be 1.6-fold higher than that in aerated solution.

The quantum yields of splitting of **1B** and **1D** were found to increase with increasing concentration of dimer. At the highest concentration of dimer used (1.2×10^{-3} M), Φ_{spl} was found to be 1.5 for dimer **1B** and 0.35 for dimer **1D**. Unlike the results typically observed in a sensitization experiment, a plot of Φ^{-1} vs [dimer]$^{-1}$ was *non*linear for both dimers. It was possible, however, to fit a curve to the data on the basis of the mechanism in Scheme I (see Discussion). Plots of the theoretical curve and the experimental data are shown in Figure 2. Dimers **2** and **3**, in which a trimethylene bridge links the dimer at N(1) and N(1'), were found to split with much lower quantum yields, as shown in Table I.

The fluorescence emission of ac_4rfH^+ in acidified acetonitrile at room temperature had λ_{max} = 507 nm. Fluorescence emission was measured in the presence of varying amounts of dimer. As dimer concentration increased, the fluorescence intensity was found to decrease, and the spectrum exhibited a slight, gradual blue shift in λ_{max} (to 495.5 nm at [**1B**] = 4.28 mM). A Stern–Volmer plot of F°/F vs [**1B**] (up to ~4 mM) and [**1D**] (up to ~20 mM) gave $k_q\tau$ as the slopes of these lines, which were 1.1×10^2 M^{-1} for **1B** and 2.0×10^1 M^{-1} for **1D**. From these values and τ = 4.8 ns (see below), k_q can be estimated at 2.3×10^{10} and 4.1×10^9 M^{-1} s^{-1} for **1B** and **1D**, respectively.

It was found that small amounts of added water caused an increase and a red-shift in fluorescence intensity, effectively abolishing fluorescence quenching by dimers **1B** and **1D**. Addition of 0.09 M H_2O increased tetraacetylriboflavin fluorescence by 19%, 0.19 M gave a 38% increase, and 0.28 M gave a 61% increase. The fluorescence lifetime of the protonated flavin was found to be 4.8 ns by time-resolved fluorescence measurements. The lifetimes in the presence of various concentrations of dimer

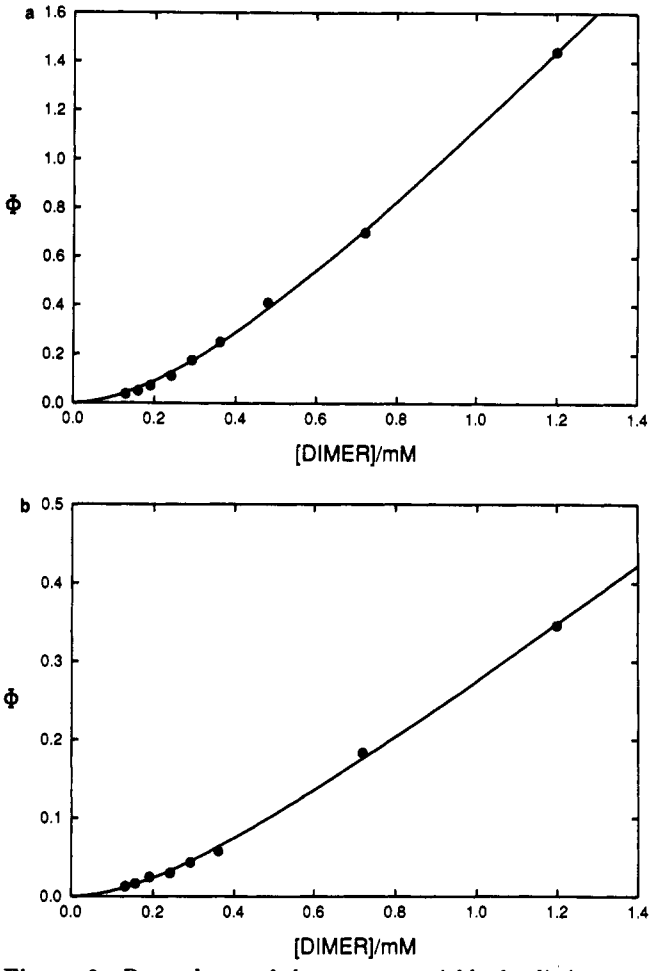

Figure 2. Dependence of the quantum yield of splitting on concentration of dimer (a) **1B** or (b) **1D**. The lines are calculated on the basis of an expression derived for the mechanism in Scheme I by use of the steady-state approximation. The expression is $\Phi_{spl} = [D]^2/[[k_t(k_f + k_{isc})[D]/\beta k_{isc}k_M] + [k_s k_t(k_f + k_{isc})/\beta k_s k_{isc}k_M]]$, where the rate constants are as shown in Scheme I, except k_t replaces FlH^*. This expression takes the algebraic form $y = x^2/(ax + b)$ for the purposes of curve fitting.

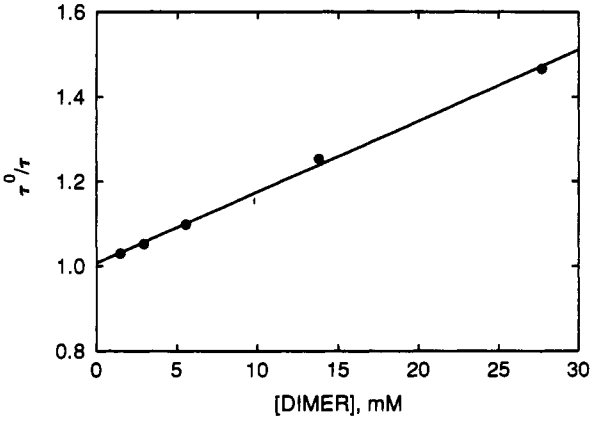

Figure 3. The dependence of the ratio of the fluorescence lifetimes τ^0/τ of ac_4rfH^+ on [**1D**] is presented in this Stern–Volmer plot. The least-squares line had slope = 16.8 M^{-1} and y-intercept = 1.007.

Table II. Quantum Yield of Splitting (Φ_{spl}) of Compound 4 Irradiated at 436 nm

condns	Φ_{spl}	condns	Φ_{spl}
aerobic	0.029	oxygen-saturated	0.023
oxygen-free	0.036		

1D were measured. A Stern–Volmer plot of the ratio of the lifetimes in the absence (τ^0) and presence (τ) of dimer against dimer concentration is shown in Figure 3.

Irradiation of ac_4rfH^+ in the presence of linoleic acid in deaerated acetonitrile/$HClO_4$ is known to result in efficient photoreduction to the radical $ac_4rfH_2^{\bullet+}$. This species has a characteristic broad absorption band at 480–500 nm and is fairly stable toward O_2 (10 h required for complete oxidation, according to the literature[9c]). Dimer **1D** was added to a freshly generated solution of $ac_4rfH_2^{\bullet+}$, and irradiation was carried out for 1 h at 496 nm. No splitting could be detected by NMR spectroscopy.

Compound **4**, in which the dimer and flavin are covalently bound, also was protonated in acidified acetonitrile (λ_{max} = 370, sh 420 nm). Irradiation at 436 nm resulted in splitting of the dimer into the corresponding pyrimidines, as evidenced by the increase in UV absorbance at 280 nm (data not shown). Thymine and orotate did not exhibit the acid-induced red-shift that is observed with 1,3-dimethyluracil. The quantum yields of splitting of compound **4** in aerated, oxygen-free (nitrogen-purged for 20 min), and oxygen-saturated (O_2-purged for 3 min) solutions are shown in Table II. Volume changes due to purging were less than 5%. The fluorescence of compound **4**, in which dimer and flavin are covalently bound, was found to be 34% lower than that of tetraacetylriboflavin (data not shown). Upon addition of water, the emission from **4** dramatically increased and was shifted to longer wavelengths.

Discussion

In acetonitrile containing perchloric acid, ac_4rf is protonated, as evidenced by the appearance of a new absorption band at 390 nm. Irradiation of ac_4rfH^+ in the presence of *cis,syn*-dimethyluracil dimer resulted in efficient cycloreversion of the dimer.

Cycloreversion is photosensitized by both the singlet and the triplet flavin, as evidenced by the 1.6-fold increase in the quantum yield of splitting of **1D** observed upon exclusion of oxygen. It is likely that in the aerated solution oxygen competes with dimer for quenching the flavin triplet. Thus, the relative ratio of singlet to triplet states as inducers of splitting can be estimated at approximately 60:40. Further evidence of singlet involvement in splitting was the observed quenching of the fluorescence of ac_4rfH^+ in the presence of a high concentration of dimer in steady state experiments. Additionally, a shortening of the lifetime of the flavin excited singlet state at high dimer concentrations was observed by time-resolved fluorescence measurements. The behavior of singlet and triplet states in splitting and the detection of the radical $ac_4rfH_2^{\bullet+}$ are the subject of a laser flash photolysis study.[8b]

Abstraction of an electron from dimer by excited flavin to produce a dimer radical cation is a plausible initial step in splitting since dimer radical cations undergo efficient cycloreversion.[3b,e,g,h] The splitting efficiency of **1B** is greater than unity, which suggested a chain reaction for splitting. Furthermore, both **1B** and **1D** exhibited curved sensitization plots (i.e., Φ_{spl}^{-1} vs $[dimer]^{-1}$). A quadratic dependence of Φ_{spl} on dimer concentration (Figure 2) was found, which also suggested a chain reaction. Although preassociation of two molecules of dimer might give similar behavior, no evidence for such an equilibrium was found by high-resolution NMR spectroscopy of solutions of dimer in acidified acetonitrile.[8c]

To account for these observations, a mechanism is proposed that has as a key propagation step the abstraction of an electron from a dimer by a monomer radical cation, which is a product of dimer radical cation splitting. As shown in Scheme I, the newly formed dimer radical cation splits to form monomer and monomer radical cation. The chain reaction is terminated by the gain of an electron by the monomer radical cation in a process that competes with the propagation step. Termination may be hampered by the rapid protonation of the initially formed $ac_4rfH^{\bullet}$ to form $ac_4rfH_2^{\bullet+}$,[9] which would be expected to give up an electron to monomer radical cation less readily. An electron may eventually be returned, however, because the species $ac_4rfH_2^{\bullet+}$ did not build up in concentration except after very long irradiations (as indicated by ΔA_{490}).

From the published[16] redox potentials of dimethyluracil (1.69 V) and the cis,syn and trans,syn dimers (1.46 and 1.34 V, respectively) measured in CH_3CN without $HClO_4$, it can be calculated that oxidation of dimethyluracil dimers by dimethyluracil radical cation are spontaneous processes in the thermodynamic sense. In contrast, the redox potential of dimethylthymine is 1.45 V, compared to 1.45 and 1.35 V for *cis,syn*- and *trans,syn*-dimethylthymine dimers, respectively.[16] As seen in Table II, thymine-derived dimers are much less efficiently split than those derived from uracil, possibly due to (1) the inability of the thymine radical cation to efficiently propagate a chain reaction by oxidizing the corresponding dimer and/or (2) interference by the trimethylene bridge.

Consistent with the chain reaction hypothesis is the observation that the linked compound **4** exhibited a relatively small splitting quantum yield. This would be expected for a compound in which intramolecular charge recombination following splitting can be envisaged to readily occur in competition with the bimolecular process of chain propagation. Alternatively, the trimethylene bridge might affect dimer splitting efficiency, or a competitive process (e.g., photolytic cleavage[8d] of the benzylic ester of **4**) might be responsible for the low quantum yield observed.

The protonated form of the neutral radical (i.e., $ac_4rfH_2^{\bullet+}$), prepared by photoreduction of ac_4rfH^+ by linoleic acid in acidified acetonitrile, was unable to photosensitize detectable amounts of dimer splitting. It is known that the neutral $FADH^{\bullet}$ radical is ineffective in the enzymatic system, and protonation apparently does not alter this in the model studies carried out in acidified acetonitrile.

A chain reaction in dimer splitting has been previously demonstrated,[16] but in that case, the propagating species was a sensitizer radical cation, not the pyrimidine radical cation. Thus, sensitization plots were linear in $[dimer]^{-1}$, not quadratic. A chain *dimerization* reaction of alkenes propagated by alkene dimer radical cations is also known.[17] This process is the reverse of the reaction described here for pyrimidine dimer splitting.

In conclusion, we have shown that a protonated oxidized flavin can photosensitize splitting of pyrimidine dimers, probably via formation of the dimer radical cation. Exceptionally high quantum yields are observed in the case

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of dimethyluracil dimers, consistent with the occurrence of a chain reaction.

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Structure of Efrapeptins from the Fungus *Tolypocladium niveum*: Peptide Inhibitors of Mitochondrial ATPase[1]

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Efrapeptins, a group of peptide toxins, were isolated from the culture filtrates of the fungus *Tolypocladium niveum* and complete stereostructures of five efrapeptins C–G were established. The peptides are mitochondrial ATPase inhibitors and have insecticidal properties.

Efrapeptins are a complex mixture of peptide antibiotics produced by the fungus *Tolypocladium niveum* (syn. *Tolypocladium inflatum, Beauveria nivea*), a soil hyphomycete. The peptides are inhibitors of mitochondrial oxidative phosphorylation and ATPase activity[2] and inhibit photophosphorylation in chloroplasts.[3] Efrapeptins are catalytic site competitive inhibitors that bind to the soluble (F_1) part of the mitochondrial ATPase,[4] thus blocking accessibility of an essential arginine residue on the enzyme at the adenine nucleotide binding site.[5] Some ATPases and mutant cell lines are resistant to efrapeptins.[6] There are several reports of the use of efrapeptins as a tool for studying energy-transfer reactions in biological systems.[7] In spite of their unique biological activity, the complete structures of these peptides were not known, although a partial structure with the amino acid sequence of one of the peptides was published before.[8] In a recent communication, we reported the structure of the unusual C-terminal blocking group in efrapeptins.[1] In this paper we present the amino acid sequence of five peptides, efrapeptins C–G (1–5), along with evidence corroborating the structure of the C-terminal blocking group, a bicyclic amine that apparently plays an important role in the biological activity of efrapeptins. These peptides are rich in α-aminoisobutyric acid (Aib) and are composed of 15 amino acid residues with an acetylated N-terminus. Efrapeptins are distinct from fungal peptaibols which are α-Aib rich peptides with an acetylated N-terminus and an amino alcohol at the C-terminus.[9]

1 Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Aib-X
2 Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Iva-X
3 Ac-Pip-Aib-Pip-Iva-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Iva-X
4 Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Ala-Leu-Iva-X
5 Ac-Pip-Aib-Pip-Iva-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Ala-Leu-Iva-X
6 H-Aib-Gly-Leu-Iva-X
7 H-Pip-Aib-Gly-Leu-Iva-X
8 Ac-Aib-Gly-Leu-Iva-X
10 H-Pip-Aib-Ala-Leu-Iva-X

(1) A preliminary account of this work has been published: Gupta, S.; Krasnoff, S. B.; Roberts, D. W.; Renwick, J. A. A.; Brinen, L. S.; Clardy, J. *J. Am. Chem. Soc.* **1991**, *113*, 707. Part of this work was presented at The Second International Conference on the Biotechnology of Microbial Products: Novel Pharmacological and Agrobiological Activities; Society for Industrial Microbiology; Oct 14–18, 1990, Sarasota, FL; abstract no. p 9.

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