Photosensitized Cleavage of Thymine Dimer with Reduced Flavin: A Model for Enzymic Photorepair of DNA†

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Abstract: DNA repair enzymes (DNA photolyase) from *E. coli* and yeast contain reduced flavin ($FADH_2$) and catalyze a light-dependent monomerization of pyrimidine dimers in DNA. $FADH_2$ has been proposed as a photosensitizer. This paper describes the photosensitized monomerization of thymine dimers in the presence of reduced flavin (1-deazariboflavin, *N*-(3)-methyllumiflavin) as a model for the enzyme reactions. Oxidation of reduced 1-deazariboflavin inactivates the sensitizer, similar to that observed upon oxidation of $FADH_2$ in *E. coli* photolyase. Reduced 1-deazariboflavin is not consumed during dimer cleavage. In the initial stage of the reaction the extent of cleavage is directly proportional to the light dose. The rate of dimer cleavage is pH independent in the range pH 8.5–11.5. In the presence of excess dimer the reaction rate is directly proportional to the sensitizer concentration. The reaction exhibits hyperbolic (saturation) kinetics with respect to the dimer concentration. The quantum yield determined for the reaction with reduced 1-deazariboflavin ($\phi = 9.2 \times 10^{-3}$ at 300 nm) indicates that the model reaction is less efficient as compared with enzymic cleavage ($\phi = 1.0$).

The principal damage resulting from exposure of DNA to ultraviolet light is the formation of cyclobutane dimers between adjacent pyrimidine residues. Photoreactivating enzymes (DNA photolyases) repair UV-damaged DNA by splitting the dimers in a rather unusual reaction that requires visible light. The importance of flavin as a coenzyme in DNA photorepair has become apparent from recent studies with photolyase from *Escherichia coli*, yeast, and *Streptomyces griseus*. Oxidized flavin (a 8-hydroxy-5-deazaflavin derivative) is present in photolyase from *S. griseus*.[1] Recent studies show that the physiologically significant form of the enzyme in *E. coli* contains reduced flavin (1,5-dihydroFAD, $FADH_2$) plus a partially characterized, fluorescent, second chromophore.[2,3] Yeast photolyase also contains reduced FAD plus a second chromophore similar to the *E. coli* enzyme.[2,4]

A quantum yield of 1.0 is observed with the *E. coli* enzyme when the flavin is present as $FADH_2$.[3] When the flavin is oxidized, the enzyme is inactive.[2] A quantum yield of 1.0 means that every absorbed quantum of light is used to split dimers and that either the reduced flavin or the second chromophore can act as a sensitizer in catalysis. The second chromophore is apparently not required in the pathway involving $FADH_2$ as the sensitizer since dimer repair is observed with second-chromophore depleted enzyme. We proposed that absorption of light by $FADH_2$ results in the transfer of an electron from the excited coenzyme to the dimer, generating flavin radical plus pyrimidine dimer radical anion.[2,3] The latter is unstable[5] and spontaneously monomerizes to yield pyrimidine plus pyrimidine radical. The pyrimidine radical then donates an electron to the flavin radical, regenerating $FADH_2$.

Photosensitized cleavage of thymine dimers has been observed with a variety of compounds, including indoles[6,7] and various quinones.[8-11] In this paper we describe the photosensitized cleavage of thymine dimers with reduced flavin. This reaction provides a model for the photoreactivating enzymes from *E. coli* and yeast.

Materials and Methods

cis,syn-[*methyl*-^{3}H]Thymine dimer (2.37 μCi/μmol) was synthesized from [*methyl*-^{3}H]thymine (Research Products Int. Corp.) by a procedure similar to that described by Wulff and Fraenkel.[12] The dried product was purified by washing it with hot ethanol which selectively removes residual, unreacted thymine. Dimer concentration was determined on the basis of its absorbance at 210 nm ($\epsilon = 7600\ M^{-1}$).[13] *N*(3)-Methyllumiflavin was synthesized as described by Ghisla et al.[14] 5-Deazariboflavin was synthesized according to the method of O'Brien et al.[14] 1-Deazariboflavin and 7,8-dimethyl-1,10-ethyleneisoalloxazinium perchlorate were gifts from Dr. Wallace Ashton and Dr. Franz Müller, respectively.

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Table I. Thymine Dimer Cleavage with Various Flavins[a]

flavin	$E°'$, mV	dimer cleavage, % reduced flavin	dimer cleavage, % oxidized flavin
N(3)-methyllumiflavin	−210[c]	3.0	31.0
1-deazariboflavin	−280[d]	15.0	0
5-deazariboflavin	−273[e]	0	10.6
7,8-dimethyl-1,10-ethylene-isoalloxazinium perchlorate[b]	−22[f]	0	2.6
FAD	−209[g]		

[a] Reactions were conducted at room temperature as described in the text. Unless otherwise noted, reaction mixtures contained 1.5×10^{-4} M flavin plus 2.4×10^{-4} M *cis,syn*-[*methyl*-^{3}H]thymine dimer in 10 mM potassium phosphate buffer, pH 11.5. [b] The flavin concentration was 3.0×10^{-5}. The reaction was conducted at pH 10.5 because the compound is unstable at higher pH. [c] Reference 20. [d] Reference 21. [e] Reference 22. [f] Reference 20. [g] Reference 19.

Reaction mixtures (500 μL) containing *cis,syn*-[*methyl*-^{3}H]thymine dimer plus flavin in 10 mM potassium phosphate buffer (pH 8.5, 10.5, or 11.5) were irradiated at room temperature in specially constructed semimicro cuvettes. Samples were made anaerobic as previously described.[16] Reduced flavins were generated in situ by the addition of a small aliquot (10 μL) of dithionite. Except as indicated below, experiments were conducted with white light from a halogen lamp under conditions similar to those previously described by Jorns.[17] Dimer cleavage

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was estimated from the amount of [*methyl*-^{3}H]thymine formed. For analysis, samples were made aerobic and mixed with cold thymine (7.9×10^{-4} M). [*methyl*-^{3}H]Thymine was separated from uncleaved dimer by high-performance liquid chromatography by following a procedure similar to that described by Rokita and Walsh[11] or, in a few cases, by thin layer chromatography [ethyl acetate–1-propanol–water (4:1:2)] on silica gel 60 F_{254} plates (Merck). Fractions were mixed with ACS II scintillation fluid (Amersham) and counted on a Beckman LS 7800 liquid scintillation counter.

In quantum yield experiments, anaerobic solutions containing reduced 1-deazariboflavin (1.5×10^{-4} M) and *cis,syn*-[*methyl*-^{3}H]thymine dimer (3.6×10^{-4} M) at pH 11.5 were irradiated for varying lengths of time at 300 nm. A lamp housing (Photon Technology International Model A1000), equipped with a parabolic reflector and a 150 W xenon lamp, was used as a light source. The collimated beam was passed through a circular slit (diameter = 4.4 cm) and then filtered with use of a narrow band filter from Oriel (5.1 cm square, 10 nm band width). Samples were magnetically stirred. The light intensity (1020 erg cm^{-2} s^{-1}) was determined by chemical actionometry with use of the potassium ferrioxalate method as described by Hatchard and Parker.[18] Dimer cleavage was estimated as described above. No cleavage was detected in control samples irradiated in the absence of reduced 1-deazariboflavin.

Results and Discussion

Cleavage of Thymine Dimers: Reduced vs. Oxidized Flavins as Photosensitizers. Since the properties of enzyme-bound flavin can be significantly altered by the protein moiety,[2] our initial survey included a flavin derivative with a reduction potential similar to FAD ($E^{\circ\prime} = -209$ mV)[19] as well as derivatives with higher and lower potentials (Table I). All reactions were conducted under anaerobic conditions. Reduced flavins were generated by the addition of a small excess (10%) of dithionite. Samples were irradiated for 80 min with light from a halogen lamp. The cleavage of *cis,syn*-[*methyl*-^{3}H]thymine dimer was monitored by measuring the formation of [*methyl*-^{3}H]thymine. Among the four reduced flavins tested, the greatest extent of dimer cleavage was observed with reduced 1-deazariboflavin (Table I). Cleavage was also observed with reduced *N*(3)-methyllumiflavin whereas no cleavage was detected with the reduced form of 7,8-dimethyl-1,10-ethyleneisoalloxazinium perchlorate or with reduced 5-deazariboflavin. The latter result indicates that the cleavage observed with reduced 1-deazariboflavin is not attributable to the presence of a ribityl moiety at position 10 of the flavin and/or the presence of a slight excess of dithionite. Although certain oxidized flavins can act as a sensitizer in the splitting of thymine dimers,[11] the cleavage observed with reduced 1-deazariboflavin and reduced *N*(3)-methyllumiflavin is not due to oxidized flavin since the samples remained fully reduced throughout the irradiation period. Furthemore, no dimer cleavage is observed with oxidized 1-deazariboflavin. In the oxidized state, *N*(3)-methyllumiflavin appeared to be the most effective sensitizer, followed by 5-deazariboflavin and 7,8-dimethyl-1,10-ethyleneisoalloxazinium perchlorate, respectively (Table I). The observation that 1-deazariboflavin is active as a sensitizer only in the reduced state whereas 5-deazariboflavin is active only in the oxidized state is noteworthy since the derivatives exhibit similar 2-electron reduction potentials. However, the latter may not be relevant for reactions which depend on the photochemical properties of reduced or oxidized flavin and the corresponding 1-electron potentials.

Reduced 1-Deazariboflavin as Sensitizer: Effect of Light Dose on the Extent of Dimer Cleavage. Since 1-deazariboflavin appeared most promising as a model for *E. coli* and yeast photolyase, further studies were conducted with this analogue. The extent of dimer cleavage at pH 11.5 is dependent upon the total number of incident photons. This is evidenced by the increase in the extent of cleavage observed when the length of the illumination period is increased (Figure 1). No dimer cleavage is observed if reduced 1-deazariboflavin is omitted or if complete reaction mixtures are incubated in the dark. The observed rate of dimer cleavage is fairly linear for the first 160 min of irradiation and then curves off by 320 min when 34% of the dimers have been monomerized. The decreased rate at longer times is probably due to dimer depletion. The reaction is not subject to product inhibition as shown by the fact that reaction rates are unaffected by the addition of thymine. The decreased rate observed after prolonged irradiation is also not due to decomposition of the sensitizer. Reduced 1-deazariboflavin is quite stable toward light, as shown by the following observations: (1) Irradiation does not affect the absorption spectrum of reduced 1-deazariboflavin. Spectra observed after reoxidation are identical with spectra observed before treatment with dithionite and light. (2) After reoxidation, irradiated samples show a single purple spot in thin-layer chromatography which comigrates with untreated 1-deazariboflavin. The quantitative recovery of the sensitizer shows that reduced 1-deazariboflavin acts as a catalyst in the reaction.

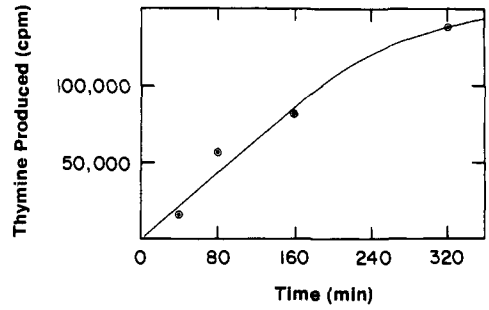

Figure 1. Effect of the length of irradiation on the extent of dimer cleavage. Samples containing [*methyl*-^{3}H]thymine dimer (2.8×10^{-4} M) and reduced 1-deazariboflavin (1.5×10^{-4} M) were irradiated under anaerobic conditions in 10 mM potassium phosphate, pH 11.5, with light from a halogen lamp and then analyzed for [*methyl*-^{3}H]thymine as described in Methods.

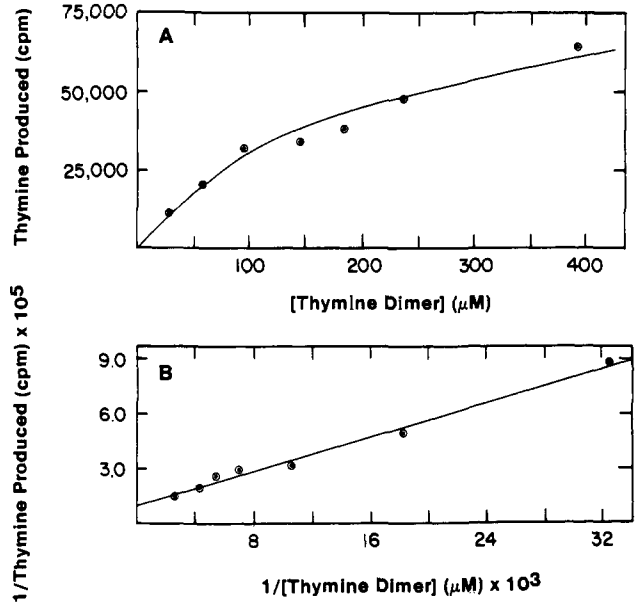

Figure 2. Effect of thymine dimer concentration on the rate of dimer cleavage. Reaction conditions are identical with Figure 1 except that the dimer concentration was varied at a constant irradiation time (80 min). In panel A, the amount of thymine formed after 80 min of irradiation is plotted as a function of dimer concentration. Panel B shows a double reciprocal plot of the data.

Effect of pH on the Rate of Thymine Dimer Cleavage. Dimer cleavage with reduced 1-deazariboflavin is also observed at pH 8.5 where it proceeds at a rate which is very similar to that observed at pH 11.5. That the reaction with reduced 1-deazariboflavin occurs at physiological pH values is quite different from the cleavage reaction observed with oxidized flavin which occurs only at alkaline pH. Protonation of thymine dimer ($pK_a = 10.7$)[13] occurs when the pH is decreased from 11.5 to 8.5. [Reduced

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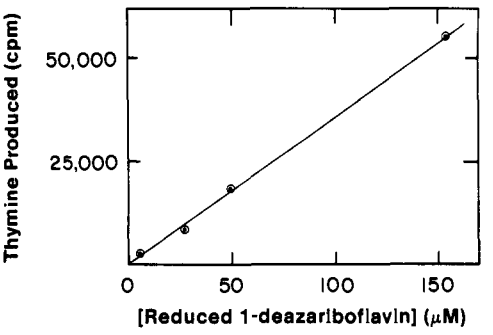

Figure 3. Effect of reduced 1-deazariboflavin concentration on the rate of dimer cleavage. Reaction conditions are identical with those of Figure 2 except that the concentration of reduced 1-deazariboflavin was varied at a constant thymine dimer concentration (3.0×10^{-4} M). The amount of thymine formed after 80 min of irradiation is plotted as a function of the sensitizer concentration.

1-deazariboflavin ($pk_a = 5.6$)[21] is unaffected]. The results indicate that the ionization state of the dimer does not affect the rate of the cleavage reaction observed with reduced 1-deazariboflavin.

Effect of Thymine Dimer Concentration on the Rate of Cleavage. As illustrated in Figure 2, the reaction observed with reduced 1-deazariboflavin as sensitizer exhibits several properties consistent with saturation kinetics: (1) At lower dimer concentrations the rate is directly proportional to dimer concentration and the reaction appears first order with respect to dimer. (2) At higher dimer concentrations the rate levels off and the kinetics approach zero order with respect to dimer. (3) A double reciprocal plot of the data is linear. The results suggest that dimer cleavage may proceed via a pathway involving excitation of a complex formed between the dimer and reduced 1-deazariboflavin, similar to that proposed[11] for the cleavage reaction observed with oxidized flavin. Tsibris et al.[23] have shown that riboflavin and thymine form a weak complex.

Effect of Reduced 1-Deazaflavin Concentration on the Rate of Dimer Cleavage. The rate of cleavage is directly proportional to the concentration of reduced 1-deazariboflavin when the latter is varied (5.6–150 μM) in the presence of excess thymine dimer (300 μM). Since none of the reaction mixtures absorbed all of the incident light, the increase in rate as a function of sensitizer concentration is expected and attributable to a proportionate increased in the concentration of the excited state of the sensitizer.

Quantum Yield. The quantum yield for dimer cleavage with reduced 1-deazariboflavin was determined at 300 nm, a wavelength where the sensitizer exhibits an absorption maximum. The observed extent of cleavage was directly proportional to the light dose in the range from 2.5×10^6 to 9.8×10^6 erg/cm^2 (data not shown). The slope of this plot was used to calculate an average value for the quantum yield ($\phi = 9.2 \times 10^{-3}$) as described by Johns.[24] The results indicate that the reaction observed with reduced 1-deazariboflavin is somewhat more efficient as compared with the cleavage observed with oxidized flavin ($\phi = 10^{-4}$–10^{-3}).[11]

Comparison of the Model Reaction with Enzymic Photorepair. The following observations indicate that the dimer cleavage observed with reduced 1-deazariboflavin provides a good model for DNA repair as catalyzed by the photoreactivating enzymes from *E. coli* and yeast: (1) Reduced 1-deazariboflavin is not consumed during dimer cleavage and hence acts as a catalyst. (2) The model reaction occurs at neutral pH. (3) Oxidized 1-deazariboflavin is inactive. *E. coli* photolyase containing oxidized FAD is also inactive.[2] (4) Both the model and the photolyase[25,26] reactions exhibit saturation kinetics. (5) Both reactions require light. In the initial stages of the reaction, the extent of dimer cleavage observed in the model and in the enzyme[25,27] reaction is a linear function of the light dose. (6) Since oxidized 1-deazariboflavin is inactive, it is unlikely that the dimer cleavage observed with reduced 1-deazariboflavin is due to an impurity. We therefore propose that reduced 1-deazariboflavin acts as a photosensitizer, similar to that proposed for $FADH_2$ in *E. coli* photolyase.[2,3] On the other hand, it should be noted that the model reaction with reduced 1-deazariboflavin ($\phi = 9.2 \times 10^{-3}$) is considerably less efficient as compared with that observed for *E. coli* photolyase containing $FADH_2$ ($\phi = 1$).[3] A similar relationship emerges when the efficiency of the model reaction with oxidized flavin ($\phi = 10^{-4}$–10^{-3})[11] is compared with that observed with *S. griseus* photolyase ($\phi = 1$),[28] a photoreactivating enzyme that uses an oxidized flavin derivative as the sensitizer.

Concluding Remarks. It has been proposed[2,3] that *E. coli* photolyase can split dimers either via a mechanism involving both $FADH_2$ and the enzyme's second chromophore or via a mechanism which requires only $FADH_2$. The model studies reported in this paper provide evidence for the feasibility of the latter mechanism. Although the enzyme contains $FADH_2$ in vivo, oxidation of $FADH_2$ to a blue neutral flavin radical occurs during enzyme isolation. Recent studies[2,3] suggest that the radical cannot act as a sensitizer and that the dimer repair observed with radical-containing enzyme is actually due to a rapid photoreduction under assay conditions which generates the active $FADH_2$ enzyme. Consistent with these observations, in model studies with a neutral flavin radical (formed from *N*(3)-methyl-*N*(5)-ethyl-1,5-dihydrolumiflavin[29]) we did not detect cleavage of thymine dimers either at neutral or at alkaline pH. Similar results have been reported with acetonitrile as solvent.[30]

The photosensitized cleavage of thymine dimers is not known to proceed via direct transfer of energy from the excited sensitizer.[11] Instead, studies with a variety of sensitizers suggest two principal modes of photosensitized cleavage: (1) electron donation from the excited sensitizer (e.g., indole derivatives) to generate thymine dimer radical anion;[6,7] and (2) electron abstraction by the excited sensitizer (e.g., oxidized flavin, various quinones) to generate thymine dimer radical cation.[8-11] Thymine dimer radicals are unstable and spontaneously monomerize. Since reduced flavin is unlikely to act as an electron acceptor, I propose that the dimer cleavage observed with reduced 1-deazariboflavin proceeds via a mechanism involving electron donation from the excited sensitizer (eq 1–4). As suggested by the observed saturation kinetics, the reaction may be initiated by the excitation of a sensitizer–dimer complex. Since the sensitizer is ionized at pH >7, the proposed electron donation step generates the known[21] zwitterionic, neutral 1-deazariboflavin radical.

$$\text{1-dFlH}^{-}\cdots\overset{\frown}{\text{TT}} \xrightarrow{h\nu} \text{1-dFlH}^{-*}\cdots\overset{\frown}{\text{TT}} \quad (1)$$

$$\text{1-dFlH}^{-*}\cdots\overset{\frown}{\text{TT}} \rightarrow \text{1-dFlH}^{\bullet} + \overset{\frown}{\text{TT}}^{\bullet-} \quad (2)$$

$$\overset{\frown}{\text{TT}}^{\bullet-} \rightarrow \text{T} + \text{T}^{\bullet-} \quad (3)$$

$$\text{T}^{\bullet-} + \text{1-dFlH}^{\bullet} \rightarrow \text{T} + \text{1-dFlH}^{-} \quad (4)$$

In contrast to the wealth of information available for oxidized flavin, very little is known about the excited states or the photochemistry of reduced flavin. Low-temperature fluorescence spectra of reduced flavins have been reported,[31] but there has been no attempt to measure phosphorescence or to characterize excited state quenchers. The cleavage of thymine dimers described in

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this paper represents one of the very few known photochemical reactions involving reduced flavin. Other examples include the photoalkylation of reduced flavin bound to lactate oxidase[32] and the photodehalogenation reactions observed with reduced 7- and 8-halogen-substituted flavins.[33]

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Communications to the Editor

Self-Reproduction of Chirality in C–C Bond Formation via Dipolar Intermediates Generated in Situ by [1,5] Hydrogen Transfer

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In this paper we describe the self-reproduction of chirality in the thermal isomerization of [[2-(1-pyrrolidinyl)phenyl]-methylene]propanedinitriles to pyrroloquinoline derivatives. During this conversion, which takes place via a 1,5-hydrogen shift and subsequent cyclization, the center of chirality in the starting material is lost in the corresponding dipolar intermediate but reproduced with retention of configuration upon cyclization. In addition the suprafacial 1,5-hydrogen shift offers the possibility to introduce a second novel chiral center with >98% enantioselectivity.

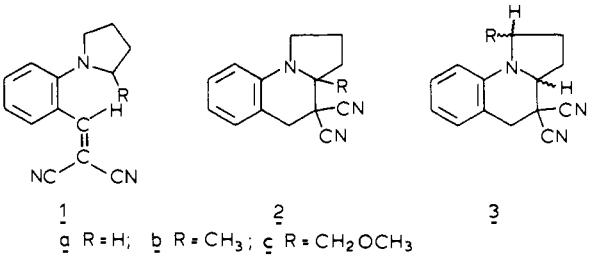
Recently, Seebach et al.[1] have described a novel method for the synthesis of chiral α-heterosubstituted carboxylic acids via α-alkylation at the chiral center. During this so-called "self-reproduction of chirality" the chirality at the reacting sp^3-C atom is temporarily lost. However, the chirality of this center is memorized by a novel chiral center that is constructed prior to reaction at the original chiral center, and conformational effects direct the alkylation of the intermediate. At the original chiral center a highly stereospecific reaction takes place.

In the course of our studies on the C–C bond formation via the "*tert*-amino effect"[2] we have previously described the thermal isomerization of [[2-(1-pyrrolidinyl)phenyl]methylene]propanedinitriles (**1a**) to pyrroloquinolines (**2a**).[2a]

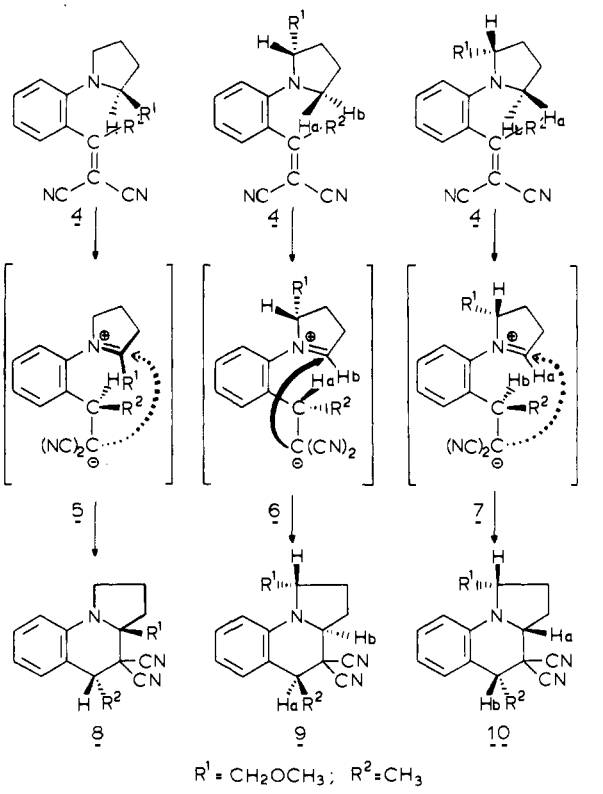
When we further studied the regioselectivity of this reaction we found that heating of **1b**[3] in refluxing 1-butanol (0.5 h) yielded selectively 1,2,3,3a,4,5-hexahydro-3a-methylpyrrolo[1,2-*a*]-quinoline-4,4-dicarbonitrile (**2b**) (85%) (Chart I). However, with a more bulky methoxymethyl substituent as *in* **1c**, the regioselectivity was lost.[3] Heating of **1c** in refluxing 1-butanol (2.5 h) gave a mixture of **2c** (46%) and two diastereomers of **3c** [(1α,3aα)-, 19%; (1α,3aβ)-, 17%].

Subsequently the stereoselectivity of the C–C bond formation was studied. Cyclization of the *chiral* (*S*)-[1-[2-[2-(methoxymethyl)-1-pyrrolidinyl]phenyl]ethylidene]propanedinitrile (**4**),[3] in which the α-carbon atom of the vinyl moiety is a *prochiral center*, in refluxing 1-butanol (5 h) gave three compounds. Compound **8**[4] was obtained in a yield of 33% together with two

Chart I



Scheme I



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(3) Compounds **1b** and **1c** were synthesized from 2-methyl- and (±)-2-(methoxymethyl)pyrrolidine, respectively, 2-fluorobenzaldehyde, and malonitrile. For the procedure see ref 2a. Compound **4** was synthesized from (*S*)-(+)-2-(methoxymethyl)pyrrolidine, 2-fluoroacetophenone, and malonitrile.[2a]

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