The λ_{max} of the glutathione adducts og β-nitrostyrene derivatives are listed in Table 4. In no case, is the a bsorption characterictics of β-nitrostyrene derivatives observed. The abscence of glutathione in the product is also determined by TLC. The Rf values of the adducts were 0.81-0.93, whereas that of glutathione is 0.58. The molecular weight determined by nonaqueous amine titration and elemental analysis results are also consistent with the proposed structure. The structure of glutathione adducts were also confirmed by NMR spectrum comparing with that of glutathione. The results are recorded in Table 5.

3-2. The effects of solvents on the addition of glutathione to β-nitrostyrene derivatives.

In an attempt to optimize the product yields, the reaction was conducted in several solvents. As shown in Figure 1, methanol gave the best results for the synthesis of 5b and 5c whereas isopropyl alcohol was the best solvent for 5a. The yield of adducts depend the solvent used and the substituted group of phenyl ring of β-nitrostyrene derivatives.

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References

(1) J. C. Sheehan and D. H. Yang, *J. Org. Chem.*, **80**, 1158 (1985).
(2) G. E. Foley, E. F. Barell, R. A. Adams and H. Lazarus, *Exp. Cell Res.*, **57**, 129(1969).
(3) K. A. Harrap and D. E. M. Speed, *Br. J. Cancer Res.*, **18**, 809(1964).
(4) K. Y. Zee-Cheng and C. C. Cheng, *J. Med. Chem.*, **13**, 414(1970).
(5) K. Y. Zee-Cheng and C. C. Cheng, *J. Med. Chem.*, **15**, 13(1972).
(6) "Glutathione, Proceedings of the symposium held at Ridgefield, Connecticu Academid Press, New York, 1954.
(7) Harington and Mead, *Biochem. J.*, **29**, 1602(1935).
(8) Hopkins, *ibid.*, 1921, **15**, 286; *J. Biol. Chem.*, **84**, 269. (1929).
(9) Kendall, Mackenzie, and Mason, *ibid.*, p.657.
(10) Du Vigneaud and Miller, (a) *J. Biol. Chem.*, **116**, 469; (1936). (b) *Biochemical preparations*, **2**, 74. (1952).
(11) Cook and Heilbron, "Chenistry of Penicillin," Princeton Univ. Press, 1949, Chap, 25.
(12) M. J. Kamlet, *J. Amer. Chem. Soc.*, **77**, 4896(1955).
(13) M. J. Kamlet and D. J. Glover, *ibid.*, **78**, 4556(1956).
(14) I. Belsky, *J. C. S. Chem. Comm.*, 237(1977).
(15) H. Hiemstra and H. Wynberg, *ibid*, 238(1978).
(16) S. Colonna and A. R. Cenro, *J, C. S. Perkin* **1**, 547(1981).
(17) Ali. Mohamed I. Abou-State, M. Amine, Hassan, N. N., El-Behairy, M. A., *Egypt. J. Chem.*, Vol. **18** (1) 55-61 (Eng.) (1976).
(18) N. Runsch, *et al., FEBS Letters*, **30**, 286(1976).
(19) M. Esterbauer, *Carbohydrate Res.*, **43**, 779(1975).
(20) I. H. Hall ,K. H. Lee, E. C. Mar and C. O. Starness, *J. Med. Chem.*, **20**, 3, 333(1977).
(21) D. E. Worral "Organic Synthesis", Col. Vol. **1**. 413, John Wiley and Sons, Inc. (1958).
(22) D. E. Wrral, *J. Amer. Soc.*, **60**, 2841(1938).
(23) E. Spath, *Monatsch.*, **40**, 129(1919).
(24) N. Cawbell, *et al. J. Chem. Soc.*, 446(1940).

Photoreaction of 8-Methoxypsoralen with Thymine

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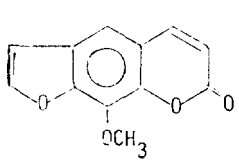
Photoreaction of 8-methoxypsoralen (8-MOP) with thymine ($\geq$ 300 nm) was carried out in the dioxane-water frozen state. One major and two minor monoaddition products between 8-MOP and thymine were isolated by various chromatographic methods. Major monoadduct was characterized to be a C_4 cycloaddition product formed between 5,6-double bond of thymine and 3,4-double bond of 8-MOP with *cis-anti* stereochemistry. Two minor adducts were proved to be stereoisomers of this major adduct.

Introduction

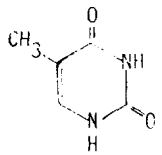
Furocoumarins, naturally occurring coumarin derivatives, are known to photoreact with pyrimidine bases, free or in DNA, upon irradiation with long wavelength UV light (320-380 nm). Various physiological actions such as skin erythema on human and guinea pig skin, mutagenic and lethal effect in bacteria, inactivation of DNA viruses, inhibition of tumor transmitting capacity of various tumor cells have been attributed to this photoreaction.[1,2] Extensive study on the skin

photosensitization ability of various furocoumarin and their molecular structure revealed that both pyrone double bond and furan double bond were required for skin photosensitizing and carcinogenic activity. The photosensitizing ability of furocoumarins is generally related to their ability to form covalent linkage with the pyrimidine bases of DNA.[3] The formation of interstrand cross linking through the C_4-photocycloaddition of 3, 4-and 4', 5'-double bond of furocoumarins to 5, 6-double bond of pyrimidine bases in DNA has been suggested as the cause of photosensitization.[4-6] When a furocoumarin is added to an aqueous solution of native DNA, molecular complexes between furocoumarin and DNA are formed by the intercalation of the planar furocoumarin molecules between the planes of two base pairs of DNA. These molecular complexes can give monofunctional or bifunctional adducts in DNA on irradiation of long wavelength UV light. The formation of both type of photoadducts indicates that cellular damage is occurring at the DNA level, and this may be the cause of photosensitization and lethality. The interstrand cross-links are believed to be largely responsible for the photosensitizing effects of psoralen treatment, although some activity is apparently associated with monoadducts.[7] There are 8 possible configurational isomers for psoralen-thymine monoadducts and 64 for biadducts. Recent NMR studies on psoralen-pyrimidine base monoadducts[8,9] and X-ray crystallography on 8-MOP-thymine monoadduct[10] have shown that biologically relevant structures have the *cis-syn* conformation. The double helical DNA conformation imposes restriction on the modes of psoralen interaction with DNA reducing the number of isomers considerably. Thus only particular conformer can be formed. Despite the extensive studies on the biological and photophysical properties of various psoralen derivatives[11] a few attempts have been made[12,13] to isolate nucleic acid-psoralen photoadducts. Recently 8-MOP-1, 3-dimethylthymine 3, 4-adducts were isolated and characterized to be a *cis-syn* and *cis-anti* isomers.[14] The *cis-syn* diastereomers of 8-MOP-thymidine 3, 4-cycloadducts from DNA were also isolated and characterized.[9] In addition to these 3, 4-cycloadducts some *cis-syn* 4', 5'-cycloadducts have been isolated and characterized between 4'-hydroxymethyl-4', 5', 8-trimethylpsoralen and thymidine, 8-MOP and thymine,[10] and between 4', 5', 8-trimethylpsoralen, 8-MOP and thymidine.[9] The isolation and structural assignment of monofunctional 5, 7-dimethoxycoumarin to thymine[15] and to thymidine[16] were also carried out. The present study is concerned with the isolation and identification of the monoadducts formed in the photoreaction of 8-MOP(1) with thymine(2) under nonbiological conditions.



8-MOP

(1)



Thymine

(2)

Materials and Methods

Materials. 8-MOP (Sigma), thymine (Sigma) and tetramethylethylene (TME. Aldrich) were used without further purification. Acetone-d_6(99.95 %), D_2O(99.95 %), DMSO-d_6 (99.9 %), pyridineed$_5$ (99.5 %) and acetic acid -d_4(99.5 %) were purchased from Aldrich. Kieselgel GF_{254}(Merck) and Kieselgel G(Merck) were used for silica gel thin layer and column chromatography. Extra pure dioxane (Wako Chemical Co) and other common solvents were used without further purification.

Spectroscopic measurements. Infrared spectra were recorded on a Perkin-Elmer-283B spectrophotometer using potassium bromide pellets. Ultraviolet-visible spectra were recorded on a Cary 17 spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian T-60A spectrophotometer and varian FT-80A spectrometer in perdeuterated pyridine, D_2O, acetone-d_6 and dimethylsulfoxide-d_6. Mass spectra were determined with Hewlett Packard 5985A GC/MS system. Fluorescence spectra were recorded on an Aminco-Bowman spectrofluorometer with Aminco XY-recorder. Elemental analyses were carried out on a F & M Scientific Cooperation -C. H. N. Analyser Model 180. The photoreaction mixtures were analyzed by reverse phase high performance liquid chromatography on a Waters' Associates Model 244 liquid chromatograph with μ-Bondapak C_{18} column.

Irradiation apparatus. Irradiations were carried out in a Rayonet Photochemical Reactor (The Southern New England Ultraviolet Company) Model RPR-208 or RPR-100 equipped with 350 nm fluorescent lamps. Two modules of model RPR-208 were stacked together and arranged in horizontal position (15 cm distance) allowing the photolysis of frozen aqueous dioxane solutions or solid state samples.

Irradiation of 8-MOP in the presence of thymine. 216 mg of 8-MOP and 1260 mg of thymine (molar ratio 1:10) were dissolved in 500 m*l* water-dioxane (2:1 v/v). The resulting solution was poured into Petri dishes and was frozen in the refrigerator. The thickness of the resulting frozen solution was less than 5 mm. The Petri-dishes containing frozen aqueous solutions were placed at 15 cm distance from the RUL-3500 Å lamps and irradiated for 20 hrs. The temperature of the reaction chamber was maintained below 0°C to prevent melting of the aqueous dioxane frozen solutions.

Analysis of photoproduct. After irradiation, the solvent was evaporated off and the residue was extracted with 100 m*l* portion of acetone to remove unreacted excess thymine. Extracted samples were analyzed by silica gel thin layer chromatography utilizing benzeneacetonitrile (55:45 v/v) as a developing solvent and visualized by mineral light. Analytical HPLC was used to analyze the photoreaction mixtures under the following conditions; column:μ-Bondapak C_{18} (3.9 mm ID$\times$30 cm), solvent: water-acetonitrile (30 : 10 v/v), flow rate: 1.0m*l*/min, detector: UV (254 nm).

Photosplitting of photoadducts. Photoadducts were dissolved in ethanol and irradiated with 2537 Å UV light in a quartz UV cell at room temperature. The UV spectra were recorded at 1 min. intervals. The light sources were RPR 2537 Å lamps

in the Rayonet photoreactor (Model RPR-100). The irradiation mixtures were analyzed by silica gel TLC.

Results and Disussion

Isolation and characterization of 8-MOP-thymine photoadducts. Isolation of 8-MOP-thymine photoadducts: The photolysis products of 8-MOP and thymine were diagnosed by TLC and three cross addition products were detected, one major and two in trace quantity. The major product (3a) was isolated by preparative TLC. When 216 mg of 8-MOP and 1260 mg of thymine were irradiated, ca 2 mg (<1 % based on 8-MOP) of the major product was obtained. A little more than 10 mg of the major photoadduct was collected by repeated experiments and used for structure determination. The minor photoadducts (3b, 3c) were formed in less than 20 % of the major photoproduct based on the HPLC analysis (Figure 1). Only a small quantity of each fraction of minor photoproducts could be obtained by utilizing semi-preparative HPLC.

Characterization of major photoadduct (3a). The elemental analysis data are consistent with the molecular formula of 1:1 adduct of 8-MOP and thymine, $C_{17}H_{14}N_2O_6$, as shown below.

Calcd for $C_{17}H_{14}N_2O_6$: C, 59.65; H, 4.08; N, 8.18; O, 28.08

Found: C, 59.68; H, 4.11; N, 7.71; O, 28.50

The mass spectra of the photoadduct were determined by electron impact (EI) and chemical ionization (CI) method. A small molecular ion peak corresponding to a 1:1 8-MOP-thymine adduct was observed at m/e 342 from the EI method and a quasi molecular ion at m/e 343 from the CI method. The fragmentation patterns show the base peak of m/e 216 (8-MOP) and relatively large peak of m/e 126 (thymine) indicating the efficient 8-MOP and thymine formation by splitting of the photoadduct. This suggests that the photoadduct is a C_4-photocycloaddition product through [2+2] addition. The low intensity of the molecular ion peak, only 2.4 % of the base peak intensity, strongly supports this proposition. The UV absorption spectrum of the photoadduct (Figure. 2) gives λ_{max} at 255 nm (275 nm shoulder), which indicates the saturation of the pyrone double bond of 8-MOP of which λ_{max} is 300 nm (330 nm shoulder). When methanol solution of the photoadduct was irradiated at 254 nm for 10 min theabsorbance at λ_{max} of 300 nm is increased considerably, indicating the photosplitting of the photoadduct into 8-MOP and thymine. When the solution irradiated at 254 nm for 10 min was subjected to TLC analysis, the spots of 8-MOP and thymine were apparent. The infrared spectra show strong carbonyl stretching band at 1710 cm^{-1} and 1745 cm^{-1}. The carbonyl stretching band of lactone was blue shifted from 1705 cm^{-1} to 1745 cm^{-1}, proving the saturation of the pyrone double bond. The other carbonyl stretching band at 1710 cm^{-1} is very close to that of thymine C_4-cyclodimer indicating the 5,6-double bond of thymine is saturated in the photoproduct. A characteristic cyclobutane ring vibrational band at 875 cm^{-1}[17] which is not observed in 8-MOP and thymine is apparent. Mass, UV absorption, and infrared spectral data along with the photosplitting experiments, strongly support that the photoadduct is a 3,4-C_4-monoadduct formed between 8-MOP and thymine. There are eight possible isomers of 3,4-photoadducts including enantiomers (*cis-syn, cis-anti, trans-syn, trans-anti*). The stereochemistry of the

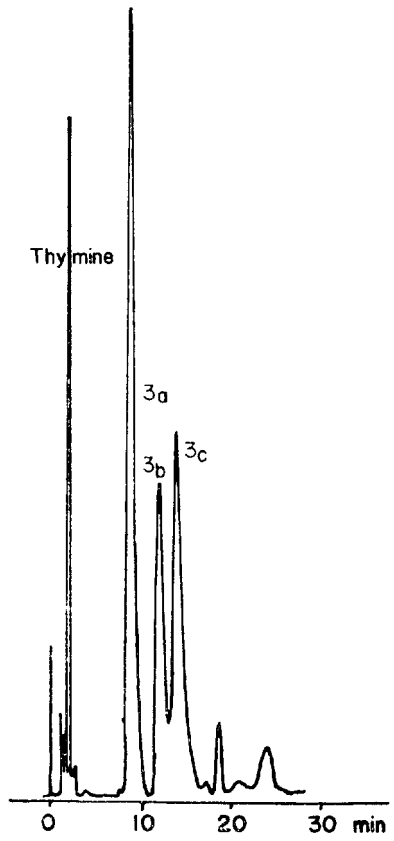


Figure 1. Liquid chromatogram of 8-MOP-thymine photoadducts.

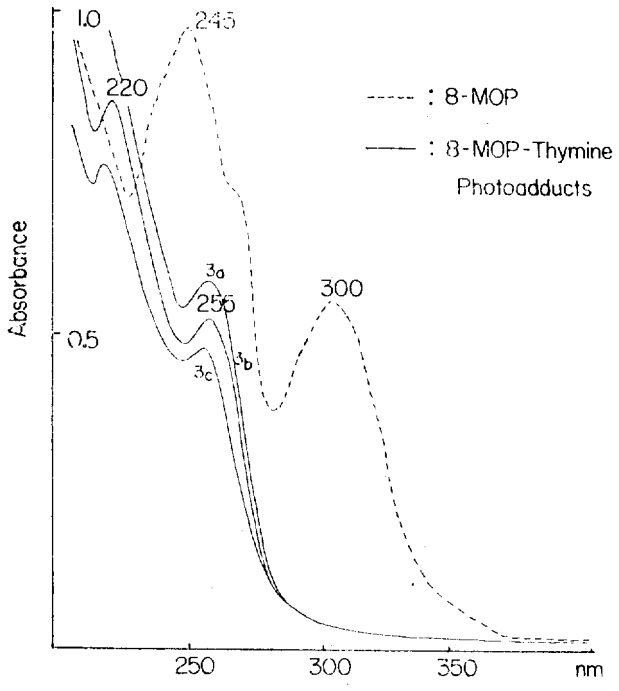


Figure 2. UV spectrum of 8-MOP-thymine photoadducts.

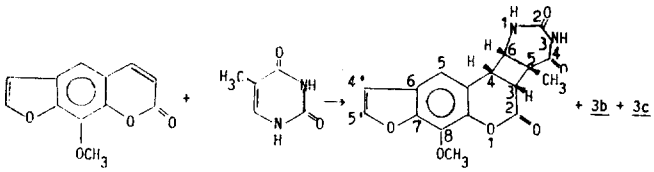
TABLE 1: Chemical Shifts (δ) and Coupling Constants (Hz) of the 8-MOP-Thymine Major Photoadduct

Assignment	Chemical shift	Multiplicity	Coupling constant (in Hz)
1-N-H (T)	9.30	d	$J_{1,6}$=3.4 Hz
5-CH_3 (T)	1.67	s	
6-H (T)	4.45	dd	$J_{4,6}$=7 Hz
			$J_{6,1}$=3.4 Hz
3-H (8-MOP)	3.75	d	$J_{3,4}$=8 Hz
4-H (8-MOP)	4.15	dd	$J_{4,3}$=8 Hz
			$J_{4,6}$=7 H z
5-H (8-MOP)	7.26	s	
8-OCH_3(8-MOP)	4.00	s	
4'-H (8-MOP)	6.67	d	$J_{4',5'}$=2 Hz
5'-H (8-MOP)	7.67	d	$J_{5',4'}$=2 Hz

photoadduct was established by ^{1}H-NMR spectra. The ^{1}H-NMR spectra of the photoadduct taken in pyridine-d_5 and in pyridine-d_5 containing one drop of D_2O show neither the pyrone double bond olefinic protons of 8-MOP at δ 6.30 and δ 7.93 nor the thymine olefinic proton at δ 6.24, again indicating the loss of the 3, 4-double bond of 8-MOP and 5, 6-double bond of thymine. 5-CH_3 protons of thymine undergo upfield shift relative to the parent compound and 5-H and 4', 5-H of 8-MOP undergo small upfield shift due to the saturation of the pyrone double bond (Table 1). New signals at δ 3.75 (d, 1H), δ 4.15 (q, 1H), and δ 4.45 (q, 1H) are observed in a spectral region typical for cyclobutane protons. The coupling (J=7 Hz) of the 4-H (δ 4.15) of the 8-MOP and the 6-H (δ 4.45) of the thymine indicates that the two protons are adjacent in the cyclobutane ring in *cis* configuration. The doublet due to 6-H of thymine is further splitted into a quartet by coupling with the adjacent 1-N-H (δ 9.30) (J=3.4 Hz). Adding one drop of D_2O to pyridine-d_5 solution of the photoadduct, the 1-N-H resonance peak was disappeared and the quartet due to the 6-H collapsed into a doublet, because of the fast exchange of 1-N-H proton with D_2O. The 4-H of 8-MOP moiety appeared as a doublet (J=8 Hz) by coupling with the adjacent 3-H of 8-MOP moiety and they are in *cis* orientation. These observations are consistent with a *cis-anti* structure of the photoadduct.

Characterization of the minor photoadducts, 3b, and 3c Due to the low concentrations, the NMR determinations of the minor photoadducts 3b and 3c could not be taken. However, UV absorption spectra of the photoadducts (see Figure 2) and their photolyzed solution at 254 nm show very close resemblance with the major photoadduct suggesting that the minor photoadducts are stereoisomers of the major photoadduct.

The mechanism of the photoaddition reaction of 8-MOP to thymine No ground state charge transfer complex is formed between 8-MOP and thymine since the absorption spectra of the mixture are exactly the sum of two individual spectra of 8-MOP and thymine. The fluorescence of 8-MOP is not quenched by thymine even though the concentration of thymine is increased up to 100 fold molar excess to 8-MOP. Therefore, no exciplex is formed between 8-MOP and thymine in contrast to 8-MOP-TME. TME quenches the fluorescence of 8-MOP, but not very efficiently (kq. τ=1.2). This observation along with the high efficiency in populating the excited triplet state of 8-MOP suggest the triplet mechanism for the photocycloaddition reaction of 8-MOP to thymine. 8-MOP photoreacts with TME giving only one 3, 4-C_4-cycloadduct probably *via* the triplet excited state as evidenced by the benzophenone sensitization. The benzophenone sensitization of the photoreaction of 8-MOP with TME enhances the quantum yield of C_4-photocycloaddition by about seven times (from 2.9×10^{-3} to 2.1×10^{-2}) suggesting the triplet mechanism for the photoaddition of 8-MOP to thymine.



3a + 3b + 3c

+ dimers of 8-MOP

Reaction condition The photoreaction of 8-MOP to thymine was carried out in aqueous dioxane frozen state under non-biological conditions. The best condition to obtain the 8-MOP-thymine photoadducts was found to be the irradiation at 350 nm of two substances in the frozen state. It has been known that the aggregate formation in aqueous solution state leads to a particular structure which imposes a restriction on the relative orientation of two neighbouring molecules. Therefore the photoreaction in aqueous frozen state limits the number of the photoadducts that can be formed and a stacked structure favours the intermolecular reaction. Thus, the photocycloaddition reaction of 8-MOP toward thymine in aqueous frozen solutions is more efficient compared with that of aqueous solution state and a major 3, 4-monoadduct with *cis-anti* configuration and two minor stereoisomers of the major photoadduct could be more easily isolated from the aqueous frozen state irradiation.

Significance of the photoadducts. The biological significance of monofunctional adducts and bifunctional adducts have been studied by many workers. The skin-photosensitizing activities of furocoumarins showed a good correlation with the number of corss-linking formed in native DNA.[4] Comparing a number of furocoumarin derivatives with various substituent groups, it was found that compounds which react most efficiently with native DNA to form cross-linkages also show the most pronounced sensitizing action on skin cells. In addition to the bifunctional adducts of furocoumarins, monofunctional adducts are able to inhibit nucleic acid synthesis[18]. Angelicin produces UV light induced monofunctional adducts with pyrimidine bases resulting in the inhibition of protein, RNA and DNA synthesis. In the photoreaction of 8-MOP with thymine, one major 3, 4-monoadduct with *cis-anti* stereoehemistry and two minor 3, 4-monoadducts were isolated. No evidence for the forma-

tion of 4′, 5′–monoadducts and biadducts could be obtained. This is in contrast to the results which were obtained in the biological conditions. Kanne et al. isolated a pair of *cis–syn* 4′, 5′–monoadducts of 8–MOP to thymidine as major adducts from the photoreaction of 8–MOP with high molecular weight, double stranded DNA. Generally it has been accepted that the pyrone double bond has higher reactivity than the furyl double bond in the triplet excited state. Under the biological conditions steric fixation of the noncovalent psoralen-DNA intercalation complex leads to the large amount of 4′, 5′–monoadducts over pyrone double bond addition products. But in the photoreaction under the nonbiological conditions this steric constraints would be decreased considerably or simply nonexistent. Thus, more reactive pyrone double bond photoreacts with thymine to give 3, 4–monoadducts.

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References

(1) M. A. Pathak, L. C. Harber, M. Seiji and, A. Kukita (Editors) "Sunlight and Man," p. 335, University of Tokyo Press, Tokyo, 1974.

(2) L. Musajo and G. Rodighiero in "Photophysiology," (Edited by C. Giese), Vol. VII, pp. 115, Academic Press, New York (1972).

(3) F. Dall'Acqua, S. M. Mango, F. Zambon and G. Rodighiero, *Photochem. Photobiol.*, **29**, 465 (1979).

(4) F. Dall'Acqua, S. Marciani, D. Vedaldi and G. Rodighiero, *Z. Naturforsch.*, **29c**, 635 (1974).

(5) R. S. Cole, *Biochim. Biophys. Acta*, **254**, 30 (1971).

(6) R. S. Cole, *J. Bacteriol.*, **107**, 846 (1971).

(7) M. L. Harter, I. C. Felkner and P.–S. Song, *Photochem. Photobiol.*, **24**, 491 (1976).

(8) K. Straub, D. Kanne, J. E. Hearst and *H.*, Rapoport, *J. Amer. Chem. Soc.*, **103**, 2347 (1981).

(9) D. Kanne, K. Straub, H. Rapoprt and J. E. Hearst, *Biochemistry*, **21**, 861 (1982).

(10) J. E. Land, F. A. P. Rushton, R. L. Beddoes, J. M. Bruce, R. J. Cernik, S. C. Dawson and O. S. Mills, *Chem. Soc. (Chem. Commun.)*, 22 (1982).

(11) P–S. Song and J. K. Tapley, *Photochem. Photobiol.*, **29**, 1177 (1979).

(12) L. Musajo, F. Bordin and R. Bevilacqua, *Photochem. Photobiol.*, **6**, 927 (1967).

(13) L. Musajo, F. Bordin, G. Caporale, S. Marciani and G. Rigatti, *Photochem. Photobiol.*, **6**, 711 (1967).

(14) B. S. Hahn, P. C. Joshi, L. S. Kan and S. Y. Wang, *Photobiochem. Photobiophys.*, **3**, 113 (1981).

(15) S. C. Shim and K. H. Chae, *Photochem. Photobiol.*, **30**, 349 (1979).

(16) S. C. Shim, H. Y. Koh and D. Y. Chi, *Photochem. Photobiol.*, **30**, 177 (1981).

(17) S. C. Shim and D. Y. Chi, *Chem. Lett.*, **11**, 1229 (1979).

(18) F. Baccichetti, F. Bordin, S. Marciani, F. Dall'Acqua and G. Rodighiero, *Z. Naturforsch.*, **31**, 207 (1976).