

ORGANIC CHEMICAL APPROACHES TO DNA PHOTOCHEMISTRY
— NEW ASPECTS OF THYMINE PHOTOCHEMISTRY —

HIROSHI SUGIYAMA

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Department of Synthetic Chemistry

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PREFACE

The study presented in this thesis has been carried out under the direction of Professor Teruo Matsuura at the Department of Synthetic Chemistry of Kyoto University during 1979 - 1983. The thesis is concerned with organic chemical approaches to DNA photochemistry involving new aspects of thymine photochemistry.

The author wishes to express his sincerest gratitude to Professor Teruo Matsuura for his constant guidance, valuable suggestions and encouragement throughout this work. The author is deeply grateful to Dr. Isao Saito for his constant advice and valuable discussions during the course of the study. The author's grateful thanks are also due to Professor Yukiteru Katsube, Osaka University and Dr. Keiichi Fukuyama, Tottori University, for their work of X-ray crystallographic analysis. The author also wishes to express his gratitude to Professor Tohru Komano and Dr. Kazumitsu Ueda, Kyoto University for sequencing of DNA. He thanks to Professor Mitsuhiro Yanagida and Messers. Yasushi Hiraoka and Shun Okada, Kyoto University for performance of the SDS gel electrophoresis and valuable discussion. The author also wishes to express his gratitude to Dr. Masahide Yamamoto, Kyoto University, for their measurements of quantum yield. Furthermore, the author wishes to thank Dr. Satoru Ito, Messors, Nobuyuki Furukawa and Takashi Morii and Akihiro Yamamoto for their collaborations. The author also wishes to thank Mr. Kenji Tanaka, Shizuoka Seiko Junior and Senior High School, for introducing him attractive field of organic chemistry.

Finally, the author express his deep appreciation to his parents and to his fiancée, Noriko Okamoto for their constant assistance and encouragements.

January, 1984.

Hiroshi Sugiyama

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General Introduction

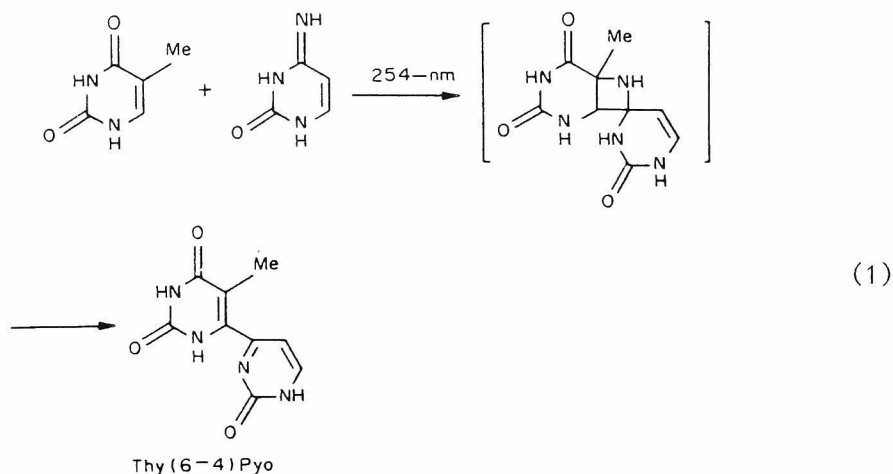
Photochemistry of DNA

UV irradiation is known to cause biological damages such as death on the cellular level and aging on the level of the organisms. Photochemistry of nucleic acids bases has been extensively studied in connection with the photobiology of nucleic acids.¹ The formation of pyrimidine photodimers and photohydrate has been recognized as one of the major reactions induced by UV irradiation of nucleic acids.¹ Numerous results on basic photoreactions of nucleic acids have been reviewed.^{1,2}

Recently DNA sequencing technique has been used to detect and measure the yields of photoproducts in DNA. Gordon et al.³ have determined the distribution of cyclobutane pyrimidine dimers in defined sequences of UV-irradiated DNA. They used a site specific cleavage of cyclobutane dimers formed in DNA by a UV-specific endonuclease.⁴ The extent of dimer formation at a potential dimer site is a function of the dose and reaches a steady-state level varying from a maximum of about 10% dimer formation at sites of adjacent cytosines.

In the course of the study concerning pyrimidine dimer formation in defined sequences, Lipple et al.⁵ have found a new type of UV-induced alkaline-labile lasions. These lesions occur at the pyrimidine sites, T-C, C-C and T-T. To examine the chemical nature of this alkaline-sensitive lesions, pyrimidine dinucleotides TpC, TpT, CpC and CpT were used as a model system.⁶ These alkaline-labile products are identified as precursors of the 6-4'-[pyrimidin-2'-one]-pyrimidine class of products that have previously been shown to occur in UV-irradiated DNA (Eq. 1). The T-T and T-C products are found in

enzymatic digests of high dose UV-irradiated salmon sperm DNA.⁶



Hall and Larcom⁷ have examined the digestion of 254-nm irradiated viral DNA with type II restriction endonucleases. UV-irradiation is shown to inhibit cleavage by nucleases which recognize sites containing thymine. Cleavage by endonucleases with other recognition sequences is not observed.

Livneh et al.⁸ have applied photoalkylation reaction with 2-propanol to synthetic deoxyribonucleotides and to various forms of ϕ X174 DNA in order to study the effect of sequences and secondary structure on the reactivity of the bases in these reactions. It is concluded that the middle purines in the sequences of Py-Pu-Py or Py-Pu-Pu are more reactive than those in the sequences of Pu-Pu-Py or Pu-Pu-Pu. The bases in the single-stranded substrates have been found to react faster than those in the double-stranded forms.

It has also been demonstrated that the exposure of DNA fragments to methylene blue and visible light causes G specific modification.⁹ Subsequent treatment with piperidine leads to chain cleavage at each G residue. The sequence of this reaction can be used for the determination of G residues in chemical DNA sequencing.

As apparent from the examples mentioned above, the molecular mechanisms of DNA photochemistry have become clearer in the past

decade. This is largely due to the development of DNA sequencing technique in combination with the accumulation of knowledge on organic photochemistry of DNA components.

Photoadditions Relevant to Nucleic Acid-Protein Photocrosslinking

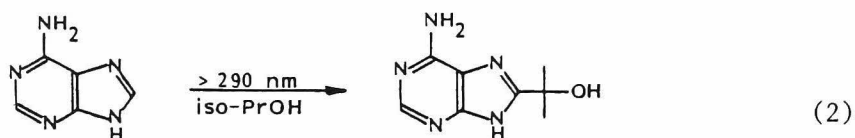
About 20 years ago Smith¹⁰ indicated that UV light could induce crosslinking of proteins to DNA in living systems. Since then there has been increasing experimental interest in photoinduced crosslinking of proteins to DNA and it has become evident that this crosslinking is an important mode of UV induced damage in biological systems. Investigation of this type of crosslinking provide important informations to elucidate not only the nature of radiation-induced damage in cells but also the structure of native DNA-protein complexes. The fact that UV light is a "Zero-Length" crosslinking reagent makes it of particular interest in determining contact points between DNA and proteins.^{2c}

For example, Schoemaker and Schimmel¹¹ have examined the UV induced crosslinking of *E. coli* tyrosyl-*t*RNA synthetase with *t*RNA^{tyr} and *t*RNA₂^{tyr} from the organism. Irradiation with a germicidal lamp led to rapid crosslinking of the RNA to proteins. Structural studies showed that only three different segments of the *t*RNA sequence were bound to the tyrosyl synthetase, indicating some degree of specificity in the crosslinking reaction. The observation that the two *t*RNA^{tyr} species were bound photochemically to different extents, although they differ by only two bases, is an indication that photo-induced crosslinking may be sensitive to slight changes in conformation of, alternatively, may depend on the bases in contact with the protein in the complex.

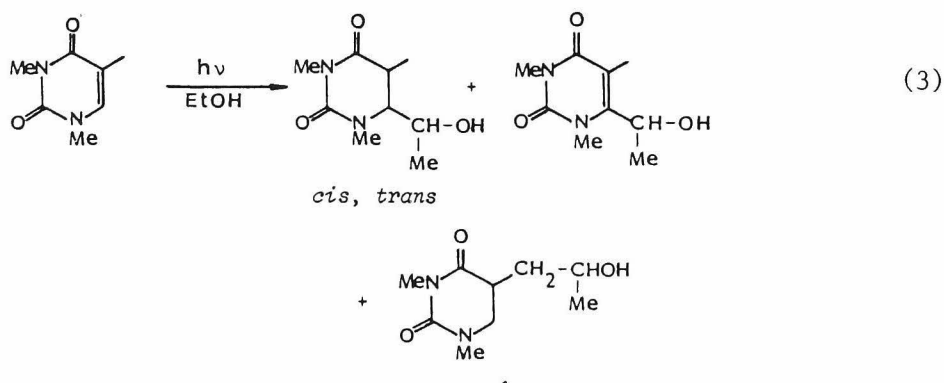
Photochemists are exploring the structures and mechanisms of formation of adducts of nucleic acid bases and related compounds with amino acids. These studies may have direct relevance to the goal of understanding of the chemical basis of UV-induced crosslinking in nucleic acid-protein complexes. Photochemical addition of

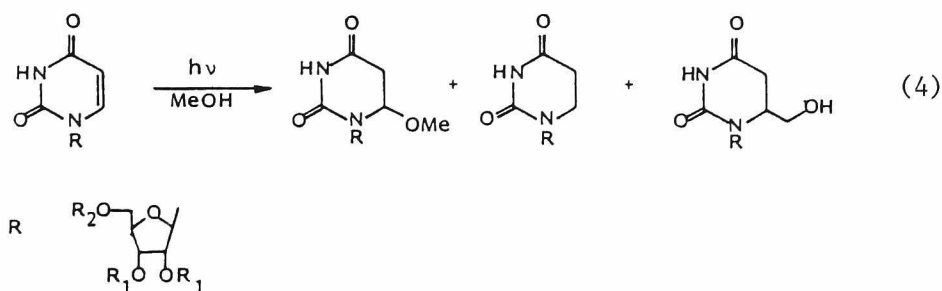
amino acids and related compounds to nucleic acid components has already been reviewed.² Therefore, the author will summarize only the recent results on the studies on model systems as below.

In connection with the photocrosslinking of serine and threonine residues of proteins to nucleic acids, photoreactions of purine and pyrimidine bases with alcohols have extensively been studied.^{2c} It has previously been demonstrated that purine derivatives react with alcohols when irradiated with UV light (> 260 nm) in the presence of a free radical photoinitiator.¹² The reaction of adenine with isopropanol is a typical example (Eq. 2).

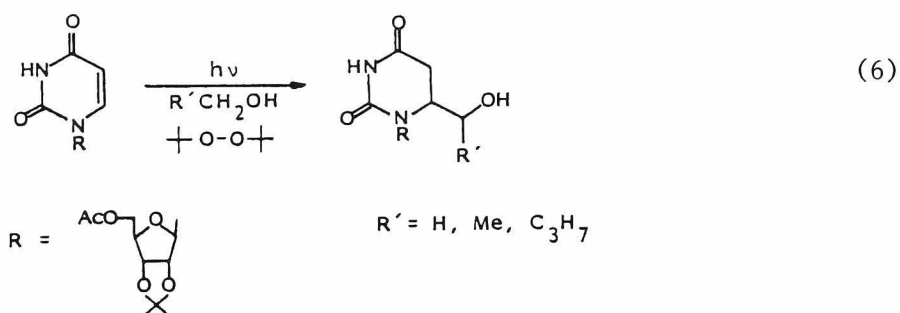
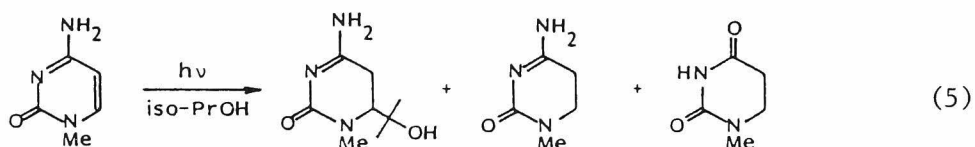


Either the direct or the acetone-sensitized irradiation of 1,3-dimethylthymine in isopropanol yields dimers of the thymine together with four products,¹³ whereas four major alcohol adducts are produced in the irradiation in ethanol (Eq. 3).¹⁴ Direct irradiation of some protected uridine derivatives in methanol leads to the formation of three products (Eq. 4)¹⁵

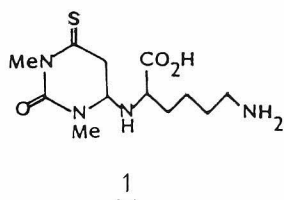
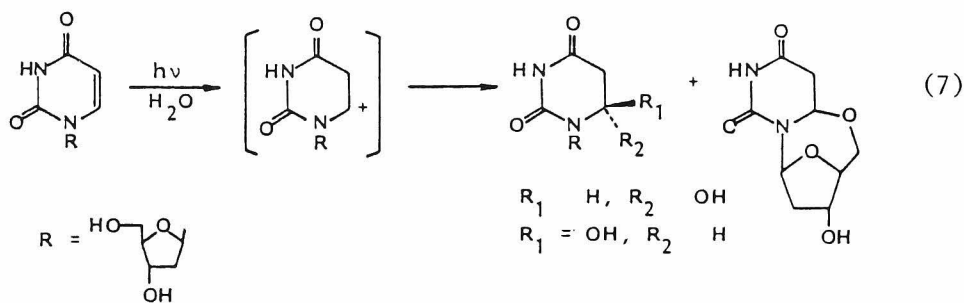




Addition of isopropanol to cytosine derivatives has also been observed together with the formation of reduction and hydrolysis products (Eq. 5).¹⁶ The same alcohol adduct is also formed by a di-*t*-butylperoxide-initiated photochemical reaction. A similar peroxide-initiated photoreaction of a protected uridine in alcohols gives synthetically useful 5,6-dihydro-6-hydroxyalkyluridines (diastereomeric mixture) in moderate yields (Eq. 6).¹⁷



The photohydration of deoxyuridine at 254-nm in aqueous solution gives the hydrate and the cyclized product probably via the common intermediate formed by the protonation of the excited molecule (Eq. 7).¹⁸ Irradiation of an aqueous solution of 1,3-dimethyl-4-thiouracil in the presence of L-lysine with a high-pressure mercury lamp through Pyrex filter gives the adduct 1.¹⁹



These studies on model systems indicate a possibility of the involvement of these photoreactions in photocrosslinking of proteins to DNA. However, direct relevance of these reactions in more complicated protein-nucleic acid system has never been elucidated.

Photocrosslinking of Histones to DNA

In eukaryotes, chromosomal DNA is packed tightly into small subunits called nucleosome.^{20a} Its structure is based upon a complex of eight histones (two each of H2A, H2B, H3, H4), which are tightly bound to each other, forming a globular core. On the outside of this histone core are firmly bound about 144 base pairs of DNA arranged in tightly superhelical coils. Model of histone core and simplified model of nucleosome are shown in Figure 1.^{20b}

An additional histone, H1, is associated more loosely with chromatin. A variety of interactions occur among the components of the nucleosome, and it is apparent that DNA-protein interactions play an important role in the structure and function of chromatin.

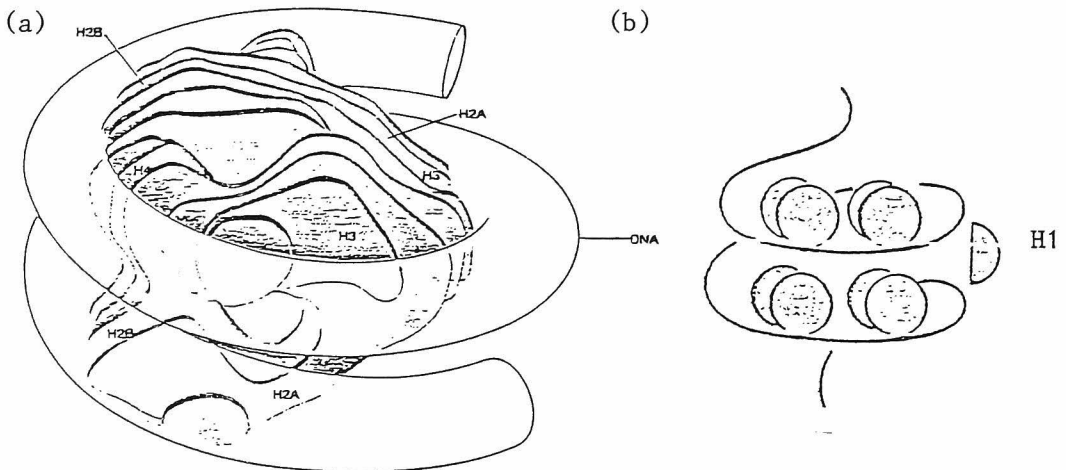


Figure 1. (a) Model of histone octamer. 144 base pairs of DNA bind on the outside of octamer.^{5d} (b) Simplified model of nucleosome.

Many reports have already dealt with the interaction between them by using chemical and physical methods.²¹ Recently several investigators have irradiated chromatin with UV light as a probe to study histone DNA interaction. Cao and Sung²² reported that cross-linked DNA-histone adducts formed by 254-nm irradiation of chicken erythrocyte nuclei were isolated and purified by hydroxylapatite chromatography, and the DNA component was subjected to acid hydrolysis. Histones isolated from hydrolyzed histone-DNA adducts were characterized by gel electrophoresis and finger print analysis. The order of observed cross-linking deduced from kinetic experiments, is H1 + H5, H3 > H4 > H2A >> H2B. The adducts produced in irradiated nuclei do not differ from chromatin, but the observed extent of histone to DNA cross-linking in nuclei is about twice that observed for chromatin. They also demonstrated that UV-induced histone-DNA cross-linking is

a sensitive conformational probe for the study of chromatin.

The amino acid sequences of the histones have largely been determined. The characterization of cross-linked histone peptides can no doubt provide a more comprehensive picture for the nature and sites of histone binding to DNA. These studies are of central importance in determining the structure and function of chromatin. Amino acid compositions of histones are given in Table 1.²³ These show that the histones are highly basic molecules with high contents of lysine and arginine. The positive charge on the side chains of lysine and

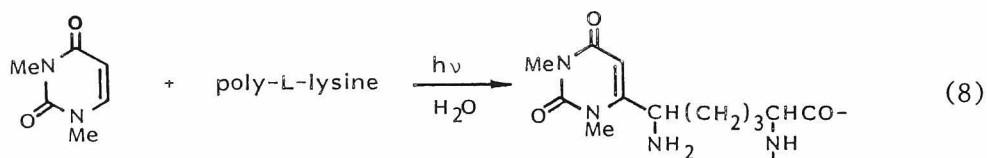
Table 1.1 Amino acid analyses (moles %) of calf thymus histone fractions and chicken erythrocyte H5. (Table provided by Dr. F. W. Johns.)

Amino acid	H2A	H2B	H3	H4	H1	H5
Asp (A)	6.6	5.0	4.2	5.2	2.5	1.7
Thr	3.9	6.4	6.8	6.3	5.6	3.2
Ser	3.4	10.4	3.6	2.2	5.6	11.9
Glu (A)	9.8	8.7	11.5	6.9	3.7	4.3
Pro	4.1	4.9	4.6	1.5	9.2	4.7
Gly	10.8	5.9	5.4	14.9	7.2	5.3
Ala	12.9	10.8	13.3	7.7	24.3	16.3
Val	6.3	7.5	4.4	8.2	5.4	4.2
$\frac{1}{2}$ -Cys	0.0	0.0	1.0	0.0	0.0	0.0
Met	0.0	1.5	1.1	1.0	0.0	0.4
Ile	3.9	5.1	5.3	5.7	1.5	3.2
Leu	12.4	4.9	9.1	8.2	4.5	4.7
Tyr	2.2	4.0	2.2	3.8	0.9	1.2
Phe	0.9	1.6	3.1	2.1	0.9	0.6
Lys	10.2	14.1	10.0	11.4	26.8	23.6
His } (B)	3.1	2.3	1.7	2.2	0.0	1.9
Arg }	9.4	6.9	13.0	12.8	1.8	12.4
Acidics (A)	16.4	13.7	15.7	12.1	6.2	6.0
Basics (B)	22.7	23.3	24.7	26.4	28.6	37.9
B/A	1.4	1.7	1.6	2.2	4.6	6.3
Lys/Arg	1.1	2.0	0.8	0.9	15.0	0.8
Gly X Arg	102.0	41.0	70.0	190.0	13.0	65.7
N-terminal group	Acetyl	Proline	Alanine	Acetyl	Acetyl	Acetyl

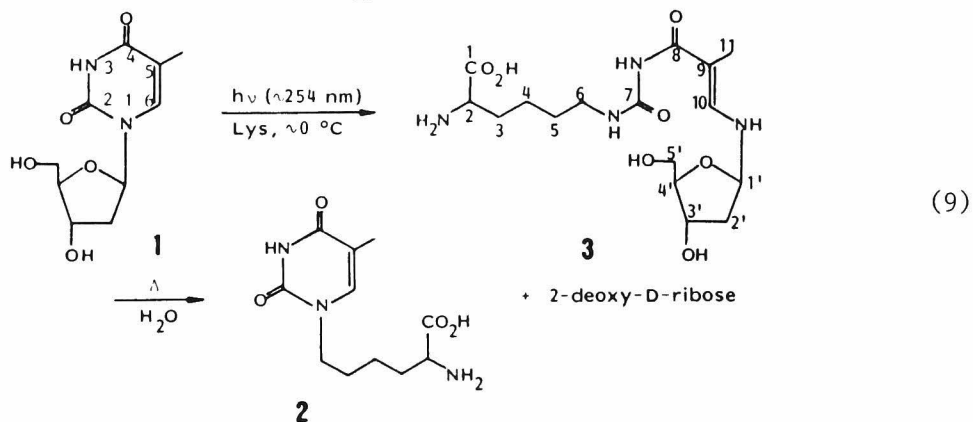
arginine at physiological pH are indicative that they are involved in the binding of proteins to the negative phosphates in the backbone of DNA. The potential proximity of these residue to the bases in DNA make these amino acids of considerable interest as participants in

UV induced DNA-histone crosslinking.

In spite of many works on model systems of photocrosslinking, our knowledge of the actual chemical nature of adducts formed in UV-irradiated DNA-histone complex is scant. Studies so far reported are as follows. Gorelic et al.²⁴ irradiated 1,3-dimethyluracil in the presence of poly-L-lysine hydrochloride at pH 10, and pH 7.4. They suggested that the 1,3-dimethyluracil was attacked to the ϵ -carbon of lysine through a bound to the 6-position of the uracil as evidenced by means of NMR analysis (Eq. 8). Shetlar et al.²⁵ recently reported that irradiation of thymine-labeled DNA and lysine in aqueous solution followed by acid hydrolysis produces a photo-product that behaves like thymine-lysine adduct, while the structure of the photoproduct has remained to be characterized.

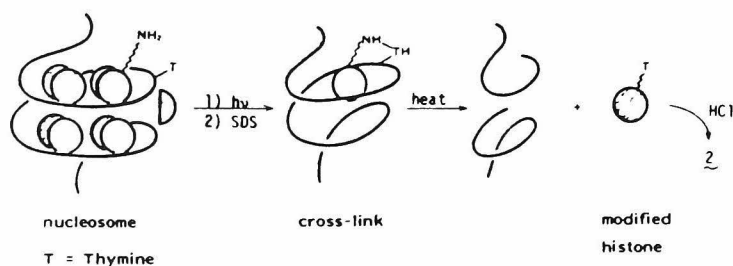


As a model for photocrosslinking of histones to DNA, we initiate to investigate the photoreaction of thymidine with lysine in aqueous solution.³¹ We have been able to reveal that lysine ϵ -amino group reacts with photoexcited Thd N(3)-monoanion to give a ring opened adduct at 0 °C which on subsequent heating leads to the formation of the thymine-lysine adduct 2 (Eq. 9). It has further confirmed that



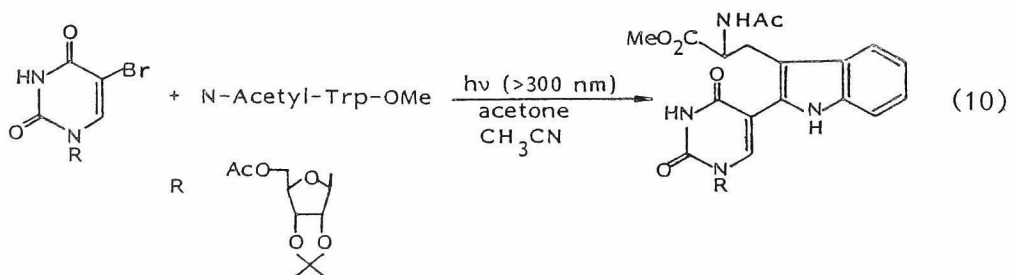
irradiation of DNA-histone complexes in calf thymus nucleohistone or in chicken erythrocyte nuclei followed by acid hydrolysis leads to the formation of the same thymine-lysine adduct 2. We have proposed the mechanism for the photocrosslinking of histones to DNA in nucleosome which is illustrated in Scheme 1. The details will be discussed in Chapter 5. However, this is one of the rare examples in which the study on model systems could directly contribute to the elucidation of the chemical mechanism of complicated protein-nucleic acid reactions.

Scheme 1. Schematic Representation of the Proposed Mechanism for Photocrosslinking in Nucleosome



Photochemistry and Stacking Interaction of Tryptophan and Pyrimidine Bases

In most of the model system concerning the photocrosslinking of protein to DNA, the absorption of light by the nucleic acid base appear to be the primary event, and very little is known about the photoreactions between nucleic acid bases and aromatic amino acids. The only study in which products have been fully characterized is that of photocoupling reaction of 5-bromouracil derivatives to tryptophan derivatives (Eq. 10).²⁶



It has also been pointed out in model compounds linked by methylene chains that a considerable amount of stacking interaction exist between indole ring and uracil ring in aqueous solution.²⁷ Helene et al.²⁸ reported that tryptophan could form complexes with pyrimidine bases in frozen aqueous solution. They proposed that aromatic amino acids could be involved in the binding of enzymes or proteins to nucleic acids by direct interaction with the bases.

Recently, Reeve and Hopkins²¹ have reported that photochemical reaction between tryptophan and pyrimidine bases. When aqueous solution of tryptophan was irradiated (> 260 nm) in the presence of uracil or thymine, two classes of photoproducts were detected. One is the dihydropyrimidine form of the bases and the other consisted of tryptophan-pyrimidine adducts, although the structures of the adducts have not been clarified. In the course of our study presented in this thesis, we investigated the stacking interaction between tryptophan and pyrimidine bases and determined the structures of tryptophan-pyrimidine photoadducts by spectral data and X-ray crystallographic analyses.³⁰

Survey of This Thesis

The present study concerning mainly with photoreaction of thymine with alkylamines has been undertaken in order to explore organic chemical basis involved in DNA photochemistry. In addition, the present study cover new aspects of thymine photochemistry which has a considerable synthetic value. Several novel photoreactions of tryptophan is also described.

Chapter 1 is concerned the photochemical reaction of thymidine with primary amines yielding ring-opened adducts. The ring-opened adducts thermally undergo ring-closure to give N(1)-alkylthymines in high yields. It is shown that a photoexcited state of Thd anion (pKa 9.8) is involved in this photoreaction.

In chapter 2, it is demonstrated that the photoaddition of thymidine to lysine and other amino acids giving rise to ring-opened

adducts. These adducts produce corresponding N(1)-substituted thymines of potent biological activities upon heating.

Chapter 3 describes a selective removal of thymine from DNA by using photoreaction with alkylamine, in which DNA is cleaved at the reacting thymines.

Chapter 4 deals with a new procedure for determining thymine residues in DNA sequencing by utilizing the photoreaction of DNA fragments in the presence of spermine.

Chapter 5 describes the isolation and characterization thymine-lysine adduct in UV-irradiated nucleohistone. A new chemical mechanism for the photocrosslinking of histones to DNA in nucleosome has been proposed.

Chapter 6 is concerned with the stacking interactions between pyrimidine base and indole ring in model compounds for nucleic acid-protein interaction.

Chapter 7 deals with a novel photochemical reaction between tryptophan and pyrimidine bases yielding a new type of adducts.

In chapter 8, it is described that a novel photochemical deuterium labeling of tryptophan at C-4 position of the indole nucleus in a highly regiospecific fashion on exposure to UV light in D₂O.

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CHAPTER 1

Photoreaction of Thymidine with Alkylamines

ABSTRACT

A new type of photoreaction between thymidine (Thd) and alkylamines has been described. Irradiation of Thd and methylamine in NaHCO_3 aqueous solution at 0 °C provided ring-opened adduct 15 quantitatively which on subsequent heating gave 1-methylthymine 8. The pH dependence of the quantum yield of the formation of ring-opened adducts suggests that a photoexcited state of Thd monoanion 19 ($\text{pK}_a = 9.8$) is involved in this reaction.

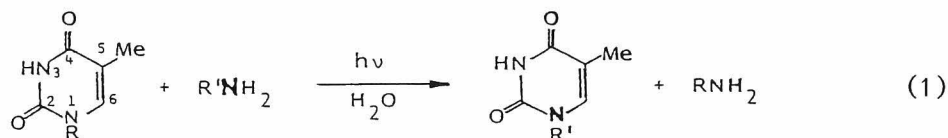
INTRODUCTION

Photochemistry of nucleic acid bases has been extensively studied for many years in connection with photobiology of nucleic acids.¹ The formation of pyrimidine photodimers and photohydrates has been recognized as one of the major reactions induced by UV irradiation of nucleic acids.¹ In view of the importance of photo-cross-linking of biopolymers, especially nucleic acid-protein crosslinks, photochemical reactions of nucleic acid components with a variety of compounds have also been thoroughly investigated.² Despite many approaches to pyrimidine photochemistry, very little is known about the photoreaction in the presence of amines.^{2c} The only study in which products have been fully characterized is that of photoreaction of 1,3-dimethyluracil with *n*-propylamine,³ although photoaddition of 4-thiouracils⁴ and free-radical initiated reaction of purines⁵ with alkylamines have previously been reported. This is surprising since basic amino groups on the side chains of lysine and arginine are suggested to be involved in the binding of proteins to the backbones of nucleic acids in nucleic acid-protein complexes, such as histone-DNA complexes,⁶ and hence photochemical reaction with amino groups is expected to play an important role in the cross-linking processes^{7,8} We therefore investigated the photochemistry of nucleic acid bases in the presence of amines in detail in order to get a clearer picture of the molecular aspects of UV induced nucleic acid-protein cross-linking.

RESULTS AND DISCUSSION

Photoreaction of Thymidine with Alkylamines. We first examined the direct irradiation of nucleosides including 2'-deoxyadenosine (dAdo), 2'-deoxyguanosine (dGuo), 2'-deoxycytidine (dCyd) and thymidine (Thd) with 254-nm light in the presence of alkylamines in aqueous solutions. Among these nucleosides Thd was the only one to react smoothly with alkylamines. For example, irradiation of aqueous solution

(pH 9.5) of Thd (1) and *n*-butylamine with a low-pressure mercury lamp through Vycor filter (mainly 254 nm) at ambient temperature ($\sim 35^\circ\text{C}$) gave rise to clean formation of 1-*n*-butylthymine (4) in 56% yield as the sole isolable product except the unreacted Thd. The existence of 2-deoxy-D-ribose (9) in the reaction mixture was confirmed by means of TLC analysis. Under similar conditions irradiation of 1 with cyclohexylamine, 2-aminoethanol and *tert*-butylamine produced the corresponding N(1)-substituted thymines 5, 6 and 7, respectively. The structures of these products were confirmed by independent syntheses.⁹ Photoreaction of simple analogues, 1-alkylthymines, with primary amines provided N(1)-substituted thymines in a similar fashion. On irradiation of 1-*n*-heptylthymine (2) and *n*-butylamine, both 4 and *n*-heptylamine were obtained in approximately the same yield. Photoreaction of 1-ethylthymine (3) with *n*-butylamine gave 4 together with liberation of ethylamine. These observations indicate that the N(1) nitrogen of 2 or 3 is extruded as N-alkylamine with the incorporation of the nitrogen of *n*-butylamine into the N(1) of the photoproduct 4. In the case of 1, 2-deoxyribose (9) is presumed to form from hydrolytically sensitive 2-deoxyribosylamine (10) under the aqueous conditions. Thus the overall process is regarded as a "photochemical exchange reaction" between Thd N(1) nitrogen and alkylamine nitrogen.



1, R β -D-deoxyribofuranosyl

2, R *n*-heptyl

3, R ethyl

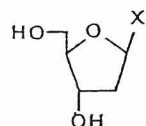
4, R' = *n*-butyl

5, R' = cyclohexyl

6, R' = hydroxyethyl

7, R' = *tert*-butyl

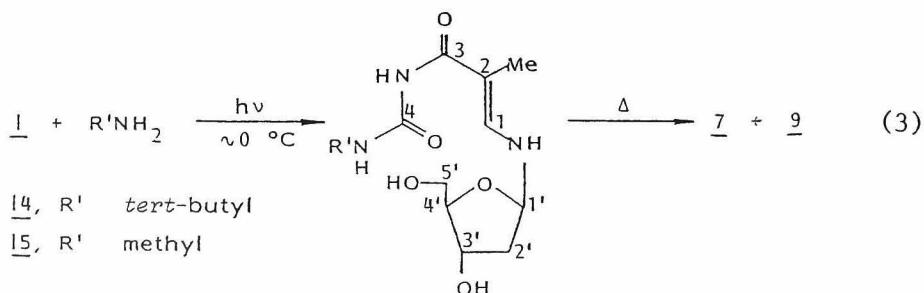
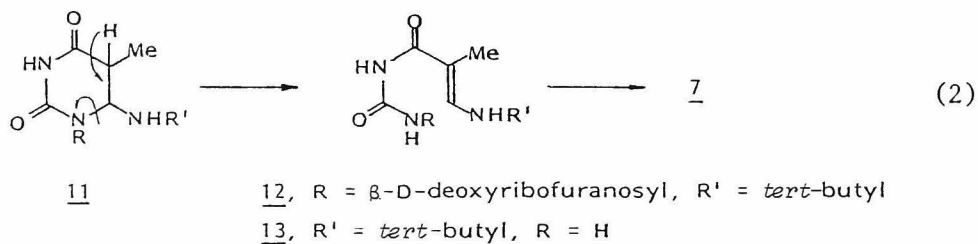
8, R' = methyl

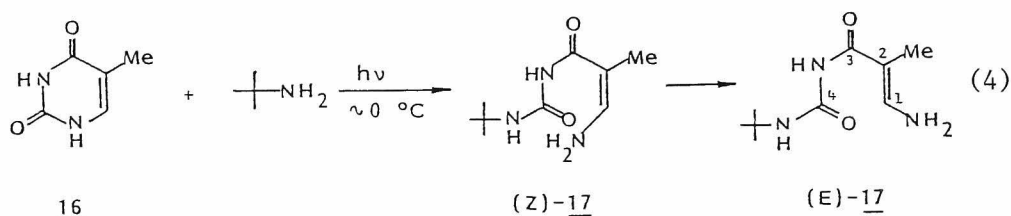


9, X = OH

10, X = NH₂

Since a variety of nucleophiles are known to attack C₆ positions of pyrimidine bases under thermal¹⁰ and photochemical conditions,^{2,11} we initially thought that nucleophilic attack of the alkylamino group on C₆ of the photoexcited 1 would first occur in this photoreaction to give 6-alkylamino-5,6-dihydrothymidine (11).⁷ Ring opening of 11 may produce 12 which on cyclization gives 7 under the basic reaction conditions. In order to get support of this hypothesis we have attempted to characterize the intermediate(s), such as 12, at low-temperature irradiation. When a solution of 1 and *tert*-butylamine in distilled water was irradiated at *ca.* 0 °C, surprisingly, ring-opened adduct 14 was obtained in 70% isolated yield in place of the expected 12. Heating of the aqueous solution of 14 rapidly produced 7 quantitatively, indicating that 14 is indeed the precursor of 7. Similarly, irradiation of thymine (16) and *tert*-butylamine at 0 °C followed by preparative high-performance LC (HPLC) at the same temperature produced a 5 : 1 E-Z mixture of 17. Irradiation of 1 and methylamine under the same conditions produced more efficiently to give less stable 15 which again transformed into 1-methylthymine (8) on subsequent heating.





The structures of these ring-opened products are evident from their spectral and chemical properties. In the ^1H NMR of (E)-17 there are an olefinic proton triplet (δ 7.48) and a two-amino proton doublet (δ 4.30), each having a large coupling constant ($J = 10$ Hz), indicating a partial structure of $>\text{C}=\text{CNH}_2$ and eliminating an alternative structure 13. The E geometry of the carbon-carbon double bond was assigned by comparison of the chemical shifts of C-methyl and olefinic protons with those of Z isomer. The olefinic protons of E isomer appeared at 0.78 ppm lower field, while the C-methyl signal being 0.05 ppm at higher field. In support of this assignment (Z)-17 gradually isomerized to more stable E isomer via enamine-imine tautomerization in alkaline aqueous solution. The ^{13}C NMR is also consistent with the assigned structure for (E)-17. The structures of both 14 and 15 were determined by their spectroscopic data including ^1H and ^{13}C NMR spectra in a similar way (Table I). In particular, the chemical shifts of C-methyl (δ 1.68) and olefinic (δ 7.36) protons in the ^1H NMR in comparison with those of (E)-17 indicate the E geometry of the carbon-carbon double bond for 14. All of the ring-opened adducts (14, 15, 17) are readily converted to the corresponding N(1)-alkylthymines upon being heated in slightly alkaline aqueous solutions. The UV absorption spectra of these products have considerably red-shifted maxima (~ 290 nm) compared to that of Thd.

Table I. ^{13}C NMR Chemical Shifts (δ , CD_3OD) for Ring-Opened Adducts **14**, **15**, and **17**

atom	14	15	(<i>E</i>)- 17 ^a
C-1	146.3	146.4	145.1
C-2	98.9	98.9	94.9
C-3	172.2	171.9	170.7
C-4	155.6	157.8	154.0
C-Me	9.9	9.9	9.0
R'	29.6, 51.6 (R' = <i>t</i> -Bu)	26.5 (R' = Me)	29.1, 49.9 (R' = <i>t</i> -Bu)
C-1'	69.6	69.5	
C-2'	35.4	35.3	
C-3'	68.5	68.4	
C-4'	85.5	85.5	
C-5'	66.9	66.7	

^a In $\text{Me}_2\text{SO}-d_6$.

Mechanistic Aspects In these bimolecular photoreaction the solution pH plays a crucial role. As shown in Figure 1, irradiation of **1** and methylamine at 0 °C did not give **15** at acidic or neutral pH, while the quantum yield of **15** increased with increasing pH in alkaline pH region (pH 8.0–11.0). The result suggests that a photoexcited state of Thd anion **19** (pK_a = 9.8) is involved in the photoaddition with methylamine. In support of this, N(3)-protected thymine such as 1,3-dimethylthymine did not undergo photoaddition with alkylamines under the conditions. Additional support for the involvement of the anion **19** is furnished by the fact that irradiation of methanolic solution of **1** in the presence of excess sodium methoxide gave a similar type of ring-opened product **18**. Irradiation of **1** and *n*-butylamine in methanol or acetonitrile never produced **4**, and **1** was recovered unchanged.

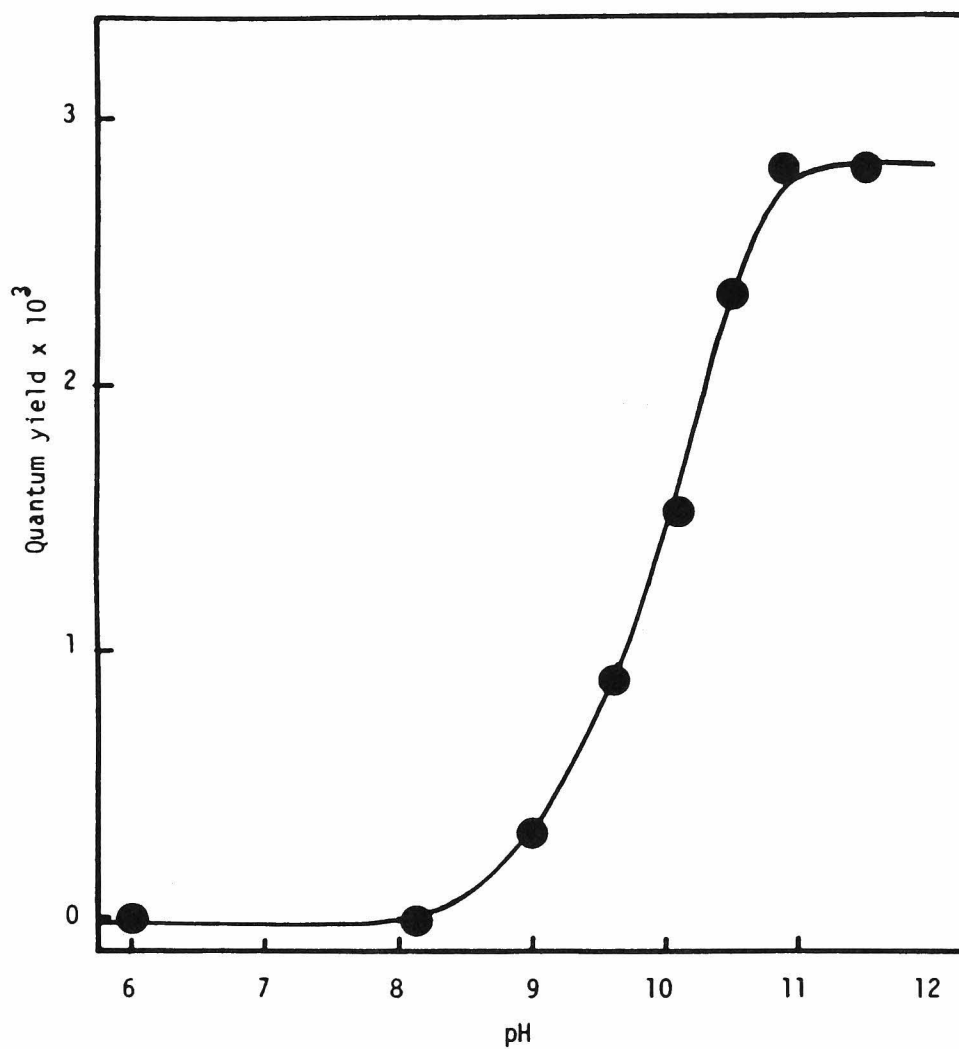
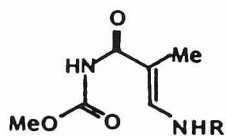


Figure 1. pH dependence of the quantum yield of the ring-opened adduct 15 in the photoreaction of Thd and methylamine at 0 °C.



18, R β -D-deoxyribofuranosyl

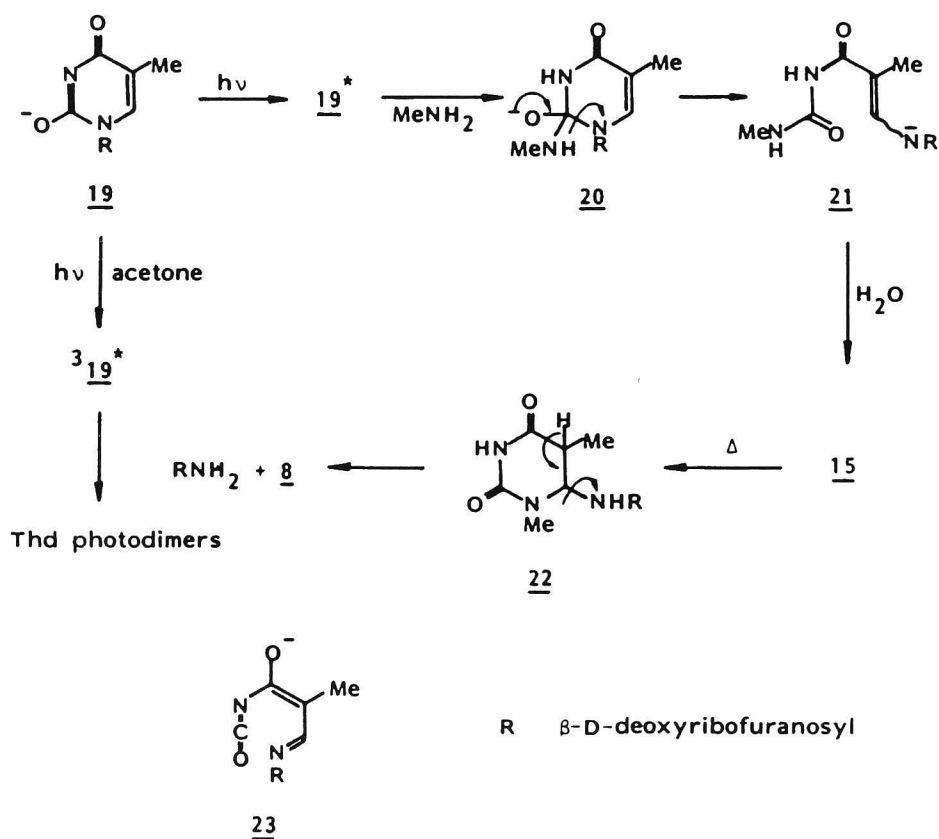
Quantum yield for the formation of the ring-opened adducts depends upon pH and amine concentrations. For example, photoreaction of 1 (0.2 mM) and methylamine (10 mM) in aqueous $\text{NaHCO}_3\text{-Na}_2\text{CO}_3$ solution at pH 10.5 gives 15 with a quantum yield of 2.4×10^{-3} (Figure 1). Thus the photoreaction with methylamine in alkaline pH region (pH 9 - 11) is an efficient process compared to the other photochemical processes reported for 1 and 16, *e.g.*, photodimerization of 1¹⁵ ($\phi = 5.6 \times 10^{-4}$) or 16¹² ($\phi = 4.7 \times 10^{-4}$) and photohydration of 16¹³ ($\phi = 3 \times 10^{-6}$) in direct irradiation in neutral aqueous solutions. In fact, there was no indication for the formation of photohydrate or photodimers of 1 in our experimental conditions.

It has been known that acetone-sensitized irradiation of thymine in aqueous solution gives a mixture of thymine photodimers via the triplet state.¹⁴ In contrast to direct irradiation leading to ring-opening reaction, acetone-sensitized irradiation of 1 in the presence of *n*-butylamine in aqueous solution never produced 4 but gave an inseparable mixture of Thd photodimers.^{14b} The result indicates that the lowest triplet state (π, π^*) of Thd anion 19 is not responsible for the photoreaction. Therefore, the most likely candidate for the excited species responsible for the formation of 4 would be either the lowest excited singlet state or a vibrationally excited ground-state of Thd anion. In view of the extremely short lifetime (a few picoseconds¹⁵) of the singlet state of 1 at room temperature together with the bimolecular nature of the photoreaction, a vibrationally excited ground-state of Thd anion rather than the singlet state is presumed to be involved in the photoreaction, although the excited species of 1 in polar protic solvents are more complicated because of the presence of tautomers¹⁶ and should await further mechanistic study.

The formation of the ring-opened adducts 14 and 15 at low-temperature irradiation clearly indicates a nucleophilic attack of alkylamines on the C_2 position of 1. We therefore propose a logical mechanism for the formation of 15 (Scheme I) by using an example of the reaction of 1 with methylamine. The first step is presumed to

involve addition of methylamine to the carbon-nitrogen double bond of photoexcited anionic species 19 to give 20. Subsequent ring-opening of 20 may yield anion 21 of greatest stabilization of negative charge by conjugation. Protonation of the anion 21 would give a E-Z mixture of the ring-opened adduct 17 as was the case of 16. In the case of 1, only the more stable E isomer 15 is isolable. Upon being heated in alkaline aqueous solution, 15 would undergo intramolecular cyclization and subsequent base-catalyzed β elimination to give 8 and 10 via 22.¹⁷

Scheme 1



Nucleophilic attack on the C₂ position of pyrimidine bases has only few precedents.¹⁸ For example, Komura et al.^{18b} have recently suggested the attack of ribose 5-phosphate anion on C₂ carbonyl of the monoanion of barbituric acid for unusual spontaneous reaction between barbituric acid and D-ribose 5-phosphate in aqueous solution. However, we are unaware of any previous example of C₂ attack of nucleophiles in the photochemistry of pyrimidine bases.² An alternative mechanism involving isocyanate 23 formed by photochemical ring-opening¹⁹ of 1 appears to be highly unlikely since aqueous solvent would intercept 23: this was not the case. Furthermore, irradiation of 1 and methylamine in methanol did not give an appreciable amount of solvent-incorporated products such as 18.

EXPERIMENTAL

Melting points are uncorrected. Elemental analyses were performed at the Analytical Center of Kyoto University. Ultraviolet spectra were recorded with a Shimadzu UV-200 spectrophotometer. ^1H - and ^{13}C NMR spectra were measured on Varian HA-100 and FT-80A spectrometers, respectively, using Me_4Si as internal standard. High-performance liquid chromatography (HPLC) was performed on a Waters ALC/GPC 204 model equipped with a 254-nm or a 280-nm fixed-wavelength detector. Reverse-phase μ -Bondapak C_{18} and Nucleosil 7C $_{18}$ (1 x 25 cm) columns were used for analytical and preparative HPLC, respectively. TLC was performed on a silica gel plate (Merck 60 PF $_{254}$).

Irradiations were carried out by using a 10 W low-pressure mercury lamp (method A) or a 100 W high-pressure mercury lamp (method B). Vycor filters were used to cut off wavelengths $\lambda < 240$ nm in all cases. For preparative runs sample solutions were irradiated internally with a 10 W low-pressure mercury lamp under nitrogen atmosphere at ambient temperature (~ 35 °C). For low-temperature irradiation the reaction vessel was cooled in a refrigerated circulating bath at 0 °C.

Following 1-alkylthymines were prepared according to the published procedures.^{9,20,21} 1-*n*-Butylthymine (4): mp 136-138 °C (lit.²² mp 140 °C); 1-cyclohexylthymine (5): mp 198-200 °C (lit.²³ mp 198 °C); 1-(hydroxyethyl)thymine (6): mp 179-181 °C (lit.²⁴ mp 179-181 °C); 1-*tert*-butylthymine (7): mp 258 °C dec, ^1H NMR (CD_3OD) δ 1.59 (s, 9 H), 1.86 (d, 3 H, $J = 1$ Hz), 7.58 (q, 1 H, $J = 1$ Hz), Anal. ($\text{C}_9\text{H}_{13}\text{N}_2\text{O}_2$) C, H, N.; 1-methylthymine (8): mp 277-280 °C (lit.²⁵ mp 281 °C).

Irradiation of Thymidine (1) and Alkylamines at Ambient Temperature. With *n*-Buthylamine. A solution of 1 (242 mg, 1 mmol) and *n*-buthylamine (219 mg, 3 mmol) in distilled water (200 mL) at pH 9.5 was irradiated at ambient temperature (~ 35 °C) under the standard conditions (method A) for 36 h. Progress of reaction was monitored by HPLC and TLC. HPLC analyses as monitored at 254 nm revealed the presence of a single product except for the starting material 1. After removal of the solvent, the residue was subjected to preparative

HPLC (1 x 25 cm Nucleosil 7C₁₈ column, methanol-water (6 : 4)). Collection of the fraction with retention time of 5 min gave 1-*n*-butylthymine (4) as colorless crystals (25 mg). ¹H NMR and chromatographic behaviors (TLC, HPLC) of the product 4 were identical with those of the authentic sample prepared independently. The yield of 4 determined by HPLC of the reaction mixture was 56% on the basis of consumed 1.

The existence of 2-deoxy-D-ribose (9) in the reaction mixture was confirmed by TLC analysis (silica gel, 2-methyl-2-propanol-AcOH-water (4 : 1 : 1)). The spot at R_f 0.53 was detected by spraying a solution of diphenylamine-aniline-80% phosphoric acid in aqueous acetone.²⁶

B. With *tert*-Butylamine. A solution of 1 (242 mg, 1 mmol) and *tert*-butylamine (730 mg, 10 mmol) in distilled water (100 mL) at pH 11.8 was irradiated under the standard conditions for 30 h. Removal of the solvent followed by preparative HPLC gave 1-*tert*-butylthymine (7) (20 mg). HPLC analysis of the mixture indicated the formation of 7 in 43% yield on the basis of consumed 1.

C. With 2-Aminoethanol. A solution of 1 (242 mg, 1 mmol) and 2-aminoethanol (366 mg, 6 mmol) in distilled water (200 mL) at pH 10.8 was irradiated for 34 h as described above. After removal of the solvent, the residue was purified by preparative HPLC (methanol-water (1 : 9)) to give 1-(hydroxyethyl)thymine (6) (32 mg). The yield of 6 was 45% on the basis of consumed 1 as determined by HPLC.

D. With Cyclohexylamine. A solution of 1 (242 mg, 1 mmol) and cyclohexylamine (297 mg, 3 mmol) was irradiated under the standard conditions for 34 h. A similar workup gave 1-cyclohexylthymine (5) as colorless crystals (41 mg). The yield of 5 was 50% as determined by HPLC.

Irradiation of 1-*n*-Heptylthymine (2) and *n*-Butylamine. A solution of 2 (89 mg, 0.4 mmol) and *n*-butylamine (219 mg, 3 mmol) in distilled water (200 mL) was irradiated at ambient temperature under the standard conditions for 60 h. HPLC analysis of the reaction mixture

as monitored at 254 nm revealed the formation of 4 in 17% yield. The existence of *n*-heptylamine in the mixture was confirmed by HPLC analysis after dansylation. To the reaction mixture (0.5 mL) were added successively Na₂CO₃ (10 mg) and 10% acetone solution of dansyl chloride (0.1 mL) and acetonitrile (1 mL), and the mixture was heated at 60 °C for 1 h. The yield of *n*-heptylamine as determined by HPLC was 16%.

Irradiation of 1-Ethylthymine (3) and *n*-Butylamine. A solution of 3 (154 mg, 1 mmol) and *n*-butylamine (219 mg, 3 mmol) in distilled water (200 mL) was irradiated under the standard conditions for 12 h. HPLC analysis of the mixture revealed the presence of 4 (90% based on consumed 1). A similar treatment with dansyl chloride followed by HPLC analysis showed the presence of ethylamine in 88% yield on the basis of consumed 1.

Isolation of Ring-Opened Adducts at Low-Temperature Irradiation.

A. Irradiation of 1 and *tert*-Butylamine. A solution of 1 (242 mg, 1 mmol) and *tert*-butylamine (730 mg, 10 mmol) in distilled water (100 mL) at pH 11.6 was irradiated at 0 °C by method A for 24 h. The resulting solution was neutralized to pH 7 with 1 N HCl and lyophilized to dryness. The residue was subjected to preparative HPLC (methanol-water (1 : 1), 2 mL/min). During elution, the solvent was cooled in ice-water bath. Two major fractions with retention times of 1.5 and 5 min were collected. The former fraction contained unreacted 1 (130 mg). The combined latter fractions were lyophilized to dryness to give 14 as hygroscopic syrup (100 mg, 70% based on consumed 1). Attempts to obtain an analytically pure sample by recrystallization were unsuccessful because of its thermal instability. Purity of 14 was more than 95% as estimated by HPLC analysis.

N-[β-(2-Deoxy-D-ribofuranosylamino)-α-methylacryloyl]-*N'*-*tert*-butylurea (14): UV (H₂O) 291 nm (log ε 4.38); ¹H NMR (CDCl₃) δ 1.37 (s, 9 H), 1.68 (s, 3 H), 2.02 (m, 2 H), 3.40-4.40 (m, 6 H), 4.70 (m, 1 H, anomeric H), 6.04 (m, 1 H, NH), 7.35 (br d, 1 H), 7.94 (br s, 1 H), 8.88 (br s, 1 H).

A solution of 14 (2 mg) in aqueous NaHCO_3 (10 mL, pH 10.5) was heated at 70 °C for 2 h. HPLC analysis of the solution revealed complete conversion of 14 to 7 (more than 98%). The existence of 9 in the solution was confirmed by TLC analysis as described before.

B. Irradiation of Thymine (16) and *tert*-Butylamine. A solution of 16 (126 mg, 1 mmol) and *tert*-butylamine (730 mg, 10 mmol) in distilled water (100 mL) at pH 11.6 was irradiated at 0 °C as described above for 24 h. The resulting solution was neutralized to pH 7.0 with 1 N HCl and lyophilized to dryness. The residue was subjected to preparative HPLC (methanol-water (1 : 9), 2 mL/min). Three major fractions with retention times of 1.6, 3, and 4.7 min were collected. The first fraction was unreacted 1 (60 mg). The second fraction was lyophilized to dryness to give (*E*)-17 as syrup (80 mg, 40% based on consumed 1).

N-[(*E*)- β -Amino- α -methylacryloyl]-*N'*-*tert*-butylurea ((*E*)-17).

UV (H_2O) 293 nm ($\log \epsilon$ 4.23); ^1H NMR (CDCl_3) δ 1.38 (s, 9 H), 1.70 (s, 3 H), 4.30 (br d, 2 H, $J = 10$ Hz, NH_2), 7.48 (t, 1 H, $J = 10$ Hz), 7.88 (br s, 1 H, NH), 8.87 (br s, 1 H, NH), Anal. ($\text{C}_9\text{H}_{17}\text{N}_3\text{O}_2$) C, H, N.

The third fraction on a similar workup gave unstable (*Z*)-17 (16 mg, 10% based on consumed 1).

N-[(*Z*)- β -Amino- α -methylacryloyl]-*N'*-*tert*-butylurea ((*Z*)-17).

UV (H_2O) 301 nm ($\log \epsilon$ 4.15); ^1H NMR (CDCl_3) δ 1.38 (s, 9 H), 1.75 (s, 3 H), 6.22 (br d, 2 H, $J = 10$ Hz, NH_2), 6.70 (t, 1 H, $J = 10$ Hz), 7.00 (br s, 1 H, NH), 8.75 (br s, 1 H, NH).

A solution of (*Z*)-17 (0.1 mg) in aqueous NaHCO_3 (pH 11.4, 10 mL) was kept at room temperature overnight. HPLC analysis indicated complete conversion of (*Z*)-17 to (*E*)-17. Heating of the solution of (*E*)-17 (0.1 mg) in aqueous NaHCO_3 solution (pH 11.0, 10 mL) at 70 °C for 2 h gave 7 in 90% yield as determined by HPLC.

C. Irradiation of 1 and Methylamine. A solution of 1 (242 mg, 1 mmol) and 40% methylamine (2 mL, 20 mmol) in distilled water (200 mL) at pH 12.0 was irradiated at 0 °C for 7 h as described above. After removal of methylamine in vacuum, the resulting solution was

neutralized and then lyophilized to dryness. Preparative HPLC (acetonitrile-water (15 : 85), 2 mL/min) of the residue at 0 °C yielded 15 (80 mg, 65% based on consumed 1). Attempts obtain an analytically pure sample were unsuccessful because of its thermal instability.

N-[(*E*)- β -(2-Deoxy-D-ribofuranosyl)amino)- α -methylacryloyl]-*N'*-methylurea ((*E*)-15): UV (H₂O) 289 nm (log ϵ 4.26); ¹H NMR (CD₃OD) δ 1.72 (s, 3 H), 1.94 (m, 2 H), 2.82 (s, 3 H), 3.40-4.40 (m, 4 H), 4.60 (m, 1 H, anomeric H), 7.43 (s, 1 H).

A solution of 15 (0.1 mg) in aqueous NaHCO₃ (pH 10.5, 10 mL) was heated at 70 °C for 2 h. HPLC analysis revealed the formation of 8 in 74% yield.

Acetone-Sensitized Irradiation of 1 and *n*-Butylamine. A solution of 1 (48 mg, 0.2 mmol) and *n*-butylamine (44 mg, 0.6 mmol) in acetone-water (1 : 3, 40 mL) was irradiated under nitrogen atmosphere with a 100-W high-pressure mercury lamp through Pyrex filter at room temperature (method B). Under this condition acetone absorbs more than 90% of the incident light in the 313-nm region. After irradiation for 10 h, the solvent was removed and the residue was analyzed by HPLC and ¹H NMR. The HPLC analysis indicated complete disappearance of 1 but none of the formation of 4. ¹H NMR of the residue showed methyl signals at δ 1.2-1.4 which are ascribable to those of isomeric Thd photodimers.¹² Further attempts to separate Thd photodimers have not been made.

Irradiation of 1,3-Dimethylthymine and *n*-butylamine. A solution of 1,3-dimethylthymine (150 mg, 1 mmol) and *n*-butylamine (439 mg, 6 mmol) in distilled water (200 mL) at pH 11.1 was irradiated by method A at ambient temperature for 48 h. HPLC analysis (methanol-water (1 : 1)) of the reaction mixture showed that it contains only the starting material.

Irradiation of 1 in the Presence of Sodium Methoxide in Methanol. A solution of 1 (242 mg, 1 mmol) and sodium methoxide (1.01g, 5 mmol) in methanol (50 mL) was irradiated at 0 °C by method A for 24 h. After addition of 0.6 N HCl (20 mL), the resulting solution was evapo-

rated to 2 mL. Preparative HPLC (methanol-water (4 : 6)) gave 18 as determined by analytical HPLC was 30% on the basis of consumed 1.

Methyl[β -((2-deoxy-D-ribofuranosyl)amino)- α -methylacryloyl]carbamate (18): UV (H_2O) 290 nm ($\log \epsilon$ 4.10); 1H NMR ($CDCl_3$) δ 1.69 (s, 3 H), 2.00 (m, 2 H), 3.20-4.20 (m, 6 H), 3.67 (s, 3 H), 4.50-4.90 (m, 2 H), 5.84 (dd, 1 H, $J = 12, 10$ Hz), 7.37 (d, 1 H, $J = 12$ Hz). Anal. ($C_{11}H_{18}N_2O_6$) C, H, N.

Quantum-Yield Measurements at Different pH. Quantum-yield measurements were carried out at 0 °C in a merry-go-round apparatus by using 5-bromouracil actinometry ($\phi = 1.8 \times 10^{-3}$).²⁷ Irradiation was made with a 400-W high-pressure mercury lamp through Vycor filter (cutoff < 240 nm). Solution of 1 (0.2 mM) and methylamine (10 mM) in 40 mM $NaHCO_3$ - Na_2CO_3 buffer (each 10 mL) were adjusted to the pH values indicated in Figure 1 in quartz tubes. For pH 6.0 the solution was acidified with 1 N HCl. The sample solutions and the actinometer solution (2×10^{-4}) were purged with nitrogen. The irradiation was stopped at less than 15% conversion of 1. The formation of 15 was assayed by UV spectroscopy monitored at 300 nm. The results are shown in Figure 1.

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CHAPTER 2

Photoaddition of Thymidine to Lysine and Other Amino Acids

ABSTRACT

Irradiation of Thd and L-lysine in aqueous solution at 0 °C provided ring-opened adduct 3 in 70% yield which on subsequent heating gave 2. Irradiation of Thd and arginine gave a different mode of adduct, (4), upon reaction with the α -amino group. Subsequent heating of 4 similarly provided 5. At strong alkaline pH (pH 11-12), irradiation of Thd and amino acids such as serine, asparagine, glycine and histidine gave the corresponding photoadducts 7, 8, 9 and 10, respectively, after heating at 70 °C for 2 h. Irradiation of Thd and peptides containing lysine residues, such as poly-L-lysine or histones, was also examined. After irradiation, the photolysates were heated at 70 °C and then subjected to acid hydrolysis (6 N HCl, 110 °C). Analysis of the hydrolysates by HPLC revealed the presence of 2 in considerable yields in both cases.

INTRODUCTION

In recent years it has become evident that photochemical cross-linking of proteins to DNA is an important process when DNA-protein complexes are irradiated with UV light.^{1,2} Investigation of this type of cross-linking is particularly useful in understanding not only the nature of radiation-induced damage in cells but also the structure of native DNA-protein complexes.

In the preceeding chapter it has been shown that primary alkylamines photochemically react with thymidine and subsequent heating of the adducts provides 1-alkylthymine. In this chapter, photoreaction between thymidine and lysine or other amino acids has been discussed. Photoreactions between thymidine and peptides containing lysine residues such as poly-L-lysine or histones were also investigated.

RESULTS AND DISCUSSION

Photoreaction of Thymidine (1) to L-lysine and L-arginine

A solution (pH 9.4) of thymidine (1, 4 mM) and free L-lysine (13 mM) in unbuffered double-distilled water was irradiated with a low-pressure mercury lamp (10 W) through Vyror filter (> 254 nm) under nitrogen atmosphere. The resulting solution was lyophilized to dryness, and the residue was subjected to preparative TLC to give a ninhydrin positive photoproduct which was further purified by preparative high-performance LC (HPLC). The yield of the photoproduct 2 was 18% on the basis of consumed thymidine. The existence of 2-deoxy-D-ribose in the photolysate was confirmed by means of TLC analysis. The R_f values of the photoproduct 2 on TLC were summarized in Table 1.

In order to characterize the precursor of 2 we have attempted Low-temperature irradiation under carefully controlled conditions. When a solution (pH 10.6) of 1 (1 mM) and free L-lysine (3 mM) in distilled water was irradiated with a 400 W high-pressure mercury lamp through Vycor filter at 0 °C for 10 h, a new UV absorption due to the formation of a ring-opened adduct appeared at around 300 nm.

Scheme 1.

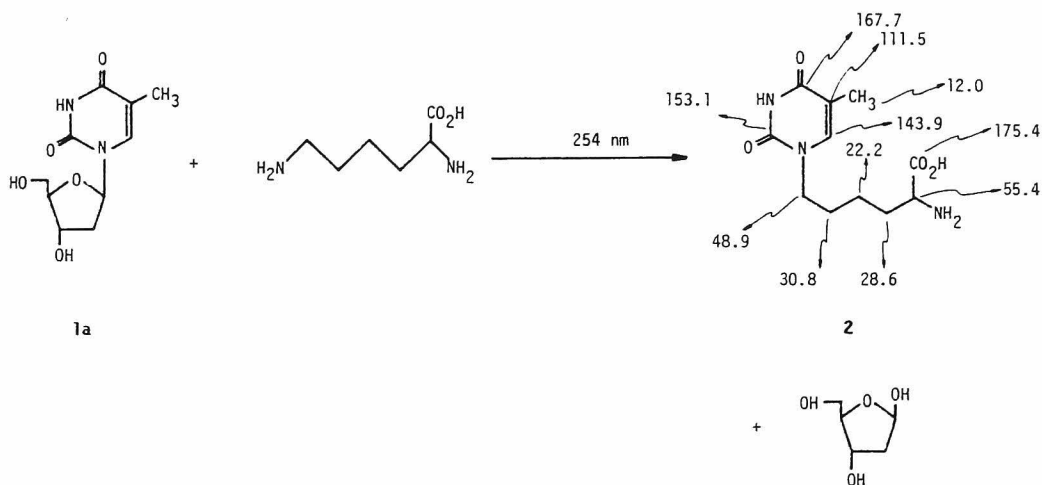


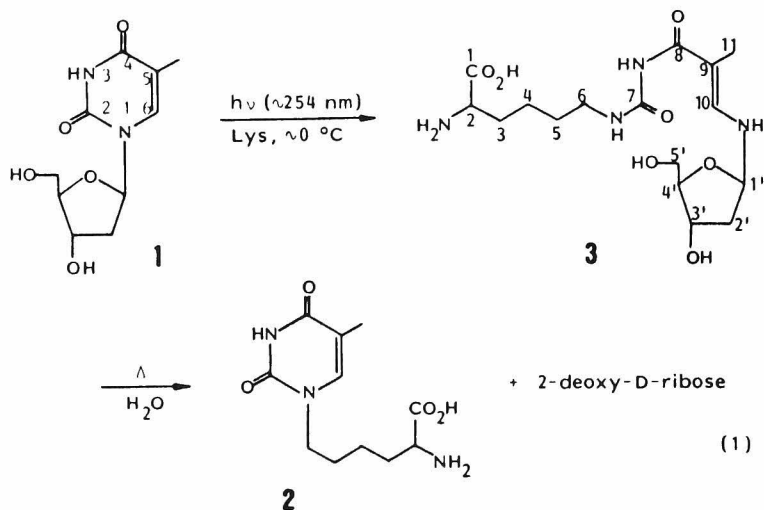
Table 1. Rf Values of Photoproduct 2

Solvent System	Silicagel (Merck 60 F ₂₅₄)	PEI-Cellulose (Merck F-PFI)
<i>t</i> -butanol-ethyl acetate-28% ammonia-water (4:3:1:2)	0.23	0.27
<i>n</i> -butanol-water (86:14)	0.06	0.12
<i>i</i> -propanol-28% ammonia-water (7:1:2)	0.60	0.42
<i>i</i> -propanol-acetic acid-water (6:3:1)	0.41	0.49

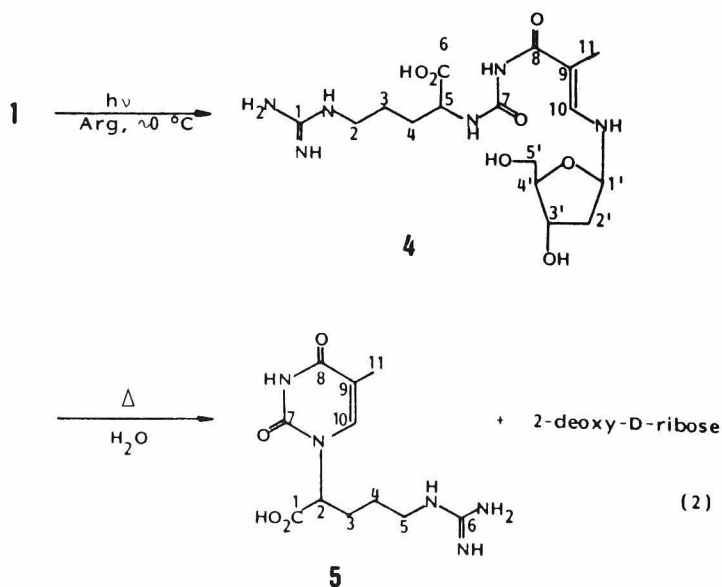
The solution was lyophilized to dryness, and the residue was subjected to preparative HPLC at temperature below 10 °C to afford 3 in 70% isolated yield on the basis of consumed 1 at 62% conversion. Heating of the aqueous solution (pH 10.5) of 3 at 90 °C instantaneously produced 2 in 90% yield together with 2-deoxy-D-ribose, indicating that 3 is indeed the precursor of 2. The compound 3 was slowly converted to 2 in neutral or alkaline aqueous solution even at room

temperature. Acidic solution of 3 rapidly produced 2 in almost quantitatively yield. Under similar conditions irradiation of 1 (0.25 mM) and free L-arginine (0.57 mM) in distilled water at pH 11.2 at 0 °C for 6 h gave a different mode of adduct, 4, in 30% isolated yield. The structure of 4 was again confirmed by converting it to 5.

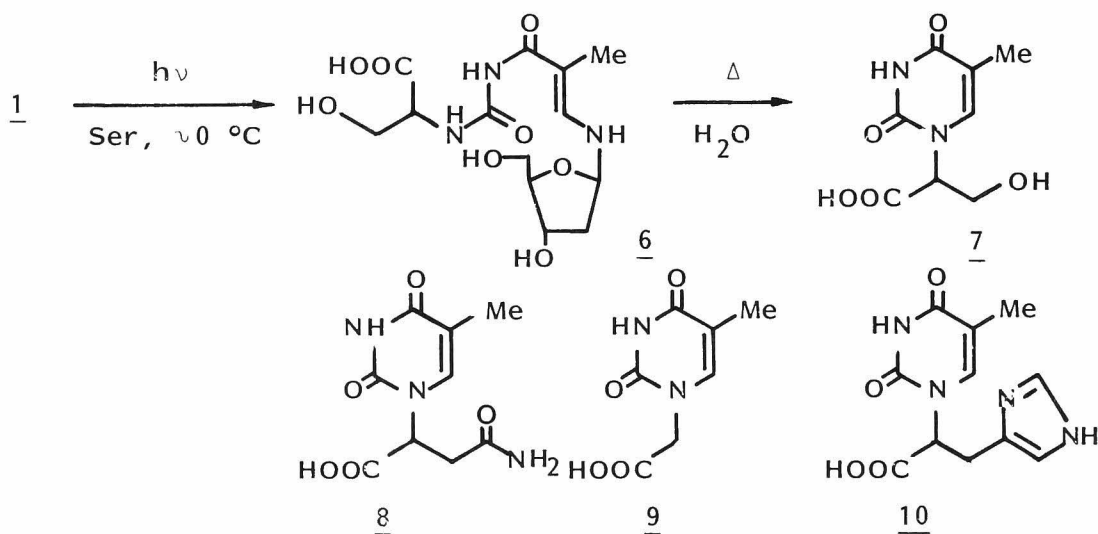
Scheme 2.



Scheme 3.



Photoreaction with Other Amino Acids. Under the condition irradiation of 1 in the presence of methylguanidine did not produce any photoproduct. Similarly, irradiation of 1 with other nucleophiles such as mercaptoethanol or imidazole did not produce any photoproduct under the irradiation condition. These results clearly demonstrated that the guanidino group of arginine, SH group of histidine, hydroxy group of serine and imidazolyl group in histidine cannot participate in the photoreaction with thymine. We next examine the photoreaction of 1 with other amino acids. Irradiation of 1 in the presence of other amino acids such as glycine and asparagine did not give corresponding photoproducts. However, at strong alkaline pH (pH 11-12) irradiation of 1 with other amino acids provided the corresponding photoproducts resulting from the reaction with α -amino groups. For example, photoreaction of 1 (2.5 mM) with serine (10 mM) in distilled water at 0 °C gave rise to 6 (30%). Upon warming at 70 °C 6 was converted to 7. Similarly, photoreaction of 1 with asparagine, glycine and histidine followed by heating gave 8 (30%), 9 (45%), 10 (30%), respectively, providing a simple method for the introduction of functional groups at N(1) position of thymine without any protection.

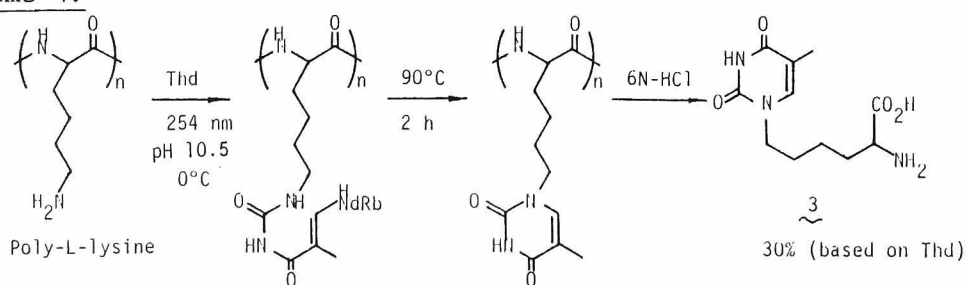


Photoreaction of 1 with Polypeptides Containing Lysine Residues

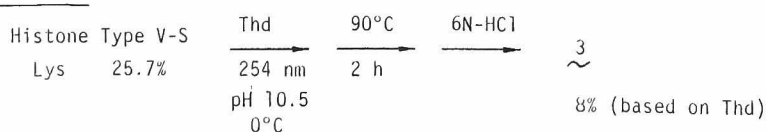
In order to ascertain whether or not this type of photoreaction occurs between 1 and lysine residue of proteins, we have examined the photoreaction of 1 with poly-L-lysine. A solution (pH 10.5) of 1 (0.2 mM) and poly-L-lysine hydrobromide (20 mg) in distilled water (5 mL) was irradiated at 0 °C under similar conditions. The photolysate was then heated at 90 °C for 2 h and was subjected acid hydrolysis (6 N HCl, 22 h, 110 °C). HPLC analysis of the hydrolysate showed the formation of 2 in 30% yield based on consumed 1 as shown in Scheme 4. Collection of the HPLC fraction corresponding to 2 gave a pure product whose chromatographic behaviors (HPLC, TLC) and UV spectrum are identical with those of the authentic sample. Likewise, irradiation of 1 with histone (Type V-S, Sigma) in distilled water (pH 10.5) followed by similar work up provided 3 in 8% yield based on consumed 1 (Scheme 5).

We next investigated the photoreaction of calf thymus DNA and poly-L-lysine. A solution (pH 10.5) of calf thymus DNA (0.3 mM) and poly-L-lysine hydrobromide (0.3 mM) in distilled water was irradiated under similar conditions. After acid hydrolysis HPLC analysis was carried out. HPLC analysis of the hydrolysate indicate the presence of 2 (0.4%) based on the thymine contained in DNA (27.6%).

Scheme 4.



Scheme 5.



Irradiation at neutral pH (pH 7.5) gave 2 only in 0.1% yield.

Such photochemical linking of lysine to 1 is of potential significance in photocrosslinking of DNA to histones in chromatin, since lysine is one of the major constituents of various histone fractions. The present observation may have a direct relevance to understand the chemical basis of UV-induced crosslinking in DNA protein complexes.

EXPERIMENTAL

Melting points are uncorrected. Elemental analysis were performed at the Analytical Center of Kyoto University. Ultraviolet spectra were recorded with a Shimadzu UV-200 spectrophotometer. ^1H and ^{13}C NMR spectra were measured on Varian HA-100 and FT-80A spectrometers, respectively, using Me_4Si as internal standard. High-performance liquid chromatography (HPLC) was performed on a Waters ALC/GPC 204 model equipped with a 254-nm or a 280-nm fixed-wavelength detector. Reverse-phase μ -Bondapak C_{18} (1 x 25 cm) columns were used for analytical and preparative HPLC, respectively. TLC was performed on a silica gel plate (Merck 60 PF₂₅₄). Ion exchange resin, Diaion HP-20 was purchased from Nippon Rensui Co. Calf thymus DNA was purchased from P-L Biochemicals and used without purification. Poly-L-lysine hydrobromide was obtained from Protein Research Foundation.

Irradiation of Thymidine (1) and L-Lysine at Ambient Temperature. A solution of 1 (100 mg, 4 mM) and L-lysine (188 mg, 13 mM) in distilled water (100 mL) at pH 9.4 was irradiated at ambient temperature ($\sim 35^\circ\text{C}$) for 48 h with a 10 W low-pressure mercury lamp through Vycor filter ($>240\text{ nm}$) under nitrogen atmosphere. The resulting solution was lyophilized to dryness, and the residues was subjected to preparative TLC (silica gel, *t*-butanol-ethyl acetate-28% ammonia-water (4:3:1:2)) to give a ninhydrin positive photoproduct which was further purified by preparative HPLC (methanol-water (1:9)).

2-Amino-6-(1-Thyminy1)hexanoic acid (2): mp $165\text{--}168^\circ\text{C}$; UV (H_2O) 270 nm ($\log \epsilon$ 3.92); ^1H NMR (D_2O) δ 1.33–1.52 (m, 2 H), 1.88 (d, 3 H, $J = 1\text{ Hz}$), 1.80–1.95 (m, 2 H), 3.73 (t, 1 H, $J = 6\text{ Hz}$), 3.78 (t, 2 H, $J = 7\text{ Hz}$), 7.50 (q, 1 H, $J = 1\text{ Hz}$); ^{13}C NMR (D_2O) δ 12.0, 22.2, 28.6, 30.8, 48.9, 55.4, 111.5, 143.9, 153.1, 167.7, 175.4. Anal. Calcd. for $\text{C}_{11}\text{H}_{17}\text{N}_3\text{O}_4 \cdot \text{H}_2\text{O}$: C; 48.34, H; 7.01, N; 15.38. Found: C; 47.91, H; 6.76, N; 15.25. The existence of 2-deoxy-D-ribose in the reaction mixture was confirmed by TLC analysis (silica

gel, *t*-butanol-acetic acid-water (4:1:1)). The spot at Rf 0.53 was detected by spraying a solution of diphenylamine-aniline-80% phosphoric acid in aqueous acetone.

Irradiation of 1 in the Presence of Nucleophiles. A solution of 1 (0.2 mM), methylguanidine (0.6 mM) and EDTA (1 mM) in 10 mM NaHCO₃-Na₂CO₃ buffer at pH 10.5 was irradiated at 0 °C for 90 min as described above. HPLC analysis (methanol-water (10:90)) of the photolysate indicated that it contains only the starting material.

Irradiation of Thymidine (1) and L-Lysine at Low Temperature. A solution of 1 (242 mg, 1 mmol) and L-lysine (439 mg, 3 mmol) in distilled water (200 mL) at pH 10.6 was irradiated with a 400 W medium-pressure mercury lamp through Vycor filter at 0 °C for 10 h. The resulting solution was neutralized to pH 7 with 1 N HCl and lyophilized to dryness. The residue was subjected to preparative HPLC (acetonitrile-water, 5:95; 4 mL/min). During elution the solvent was cooled in an ice-water bath. Two major fractions with retention times of 13 min and 26 min were collected. The former fraction contained unreacted 1 (92 mg). The combined latter fractions were lyophilized to dryness to give 3 as a hygroscopic syrup (170 mg, 70% based on consumed 1). Attempts to obtain an analytically pure sample by recrystallization were unsuccessful because of its thermal instability. Purity of 3 was more than 95% as estimated by HPLC analysis.

N-[β-(2-Deoxy-D-ribofuranosylamino)-α-methylacryloyl]-N'-(5'-amino-5'-carboxy)pentylurea (3). UV (CH₃OH) 286 nm (log ε 4.86); ¹H NMR (D₂O) δ 1.70 (s, 3 H), 1.20-2.30 (m, 8 H), 3.28 (t, 2 H, J = 6 Hz), 3.60-4.20 (m, 5 H), 4.70 (m, 1 H), 7.43 (s, 1 H); ¹³C NMR (D₂O) δ 9.3 (C-11), 22.4 (C-4), 29.0 (C-3), 30.8 (C-5), 33.5 (C-2'), 39.6 (C-6), 55.3 (C-2), 66.9 (C-1'), 67.7 (C-5'), 68.2 (C-3'), 85.3 (C-4'), 98.2 (C-9), 146.2 (C-10), 156.4 (C-7), 171.7 (C-8), 175.4 (C-1).

A solution of 3 (0.1 mg) in aqueous NaHCO₃ (pH 10.5, 10 mL)

was heating at 90 °C for 2 h gave 2 in 90% yield as determined by HPLC. The existence of deoxy-D-ribose in the solution was confirmed by TLC analysis as described before. A solution of 3 (0.1 mg) in 0.1 N HCl (pH 1.0, 10 mL) was standing at room temperature for 10 min to give 2 quantitatively.

Irradiation of 1 and L-Arginine at Low Temperature. A solution of 1 (60.5 mg, 0.25 mmol) and L-arginine (99 mg, 0.57 mmol) in distilled water (200 mL) at pH 11.2 was irradiated at 0 °C as described above for 6 h. The resulting solution was neutralized to pH 7 with 1 N HCl and lyophilized to dryness. The product was purified by chromatography on HP-20 with methanol-water (1:9) as the eluting solvent to give 4 as a colorless syrup (31 mg, 40% based on consumed 1).

N-[β-(2-Deoxy-D-ribofuranosylamino)-α-methylacryloyl]-N'-(1'-carboxy-4'-guanidino)butylurea (4). UV (H₂O) 290 nm (log ε 4.25); ¹H NMR (D₂O) δ 1.72 (s, 3 H), 1.40-2.40 (m, 6 H), 3.20 (br t, 2 H, J = 7 Hz), 3.50-4.30 (m, 6 H), 7.45 (br s, 1 H); ¹³C NMR (D₂O) δ 9.8 (C-11), 25.2 (C-3), 30.2 (C-4), 33.9 (C-2'), 41.7 (C-2), 55.8 (C-5), 67.4 (C-1'), 68.2 (C-5'), 68.7 (C-3'), 85.8 (C-4'), 98.8 (C-9), 146.8 (C-10), 156.1 (C-7), 157.6 (C-1), 172.3 (C-8), 179.3 (C-6).

A solution of 4 (0.1 mg) in aqueous NaHCO₃ (pH 10.5, 10 mL) was heating at 90 °C for 2 h to give 5 in 90% yield as determined by HPLC.

1-(1-Thyminy)-4-guanidinopentanoic acid (5). Negative ninhydrin test; UV (H₂O) 273 nm (log ε 3.91); ¹H NMR (D₂O) δ 1.27-1.55 (m, 2 H), 1.71 (d, 3 H, J = 8, 4 Hz), 7.25 (q, 1 H, J = 1 Hz); ¹³C NMR (D₂O) 12.3 (C-11), 25.7 (C-4), 28.2 (C-3), 41.2 (C-5), 61.1 (C-2), 111.5 (C-9), 141.6 (C-10), 153.3 (C-7), 157.5 (C-6), 167.2 (C-8), 176.5 (C-1). Exact mass. Found: 353.1656. Calcd. for C₁₅H₂₃O₅N₅ (monoacetyl-monomethylester) 353.1699.

Irradiation of Thymidine (1) and Other Amino Acids. A. With L-Serine. A solution of 1 (121 mg, 0.5 mmol) and L-serine (210 mg,

2 mmol) in distilled water (200 mL) at pH 12.4 was irradiated at 0 °C for 40 h with a 10 W low-pressure mercury lamp through Vycor filter (>240 nm) under nitrogen atmosphere. The resulting solution was neutralized to pH 7 with 1 N HCl and lyophilized to dryness the residue was subjected to preparative HPLC (water, 3 mL/min). Major fraction with retention times of 10 min was collected to give 6 as a colorless syrup (52 mg, 30% based on consumed 1). Attempts to obtain an analytically pure sample by recrystallization were unsuccessful because of its thermal instability. Purity of 6 was more than 95% as estimated by HPLC analysis.

N-[β-(2-Deoxy-D-ribofuranosylamino)-α-methylacryloyl]-N'-(1'-carboxy-2'-hydroxy)ethylurea (6). ^1H NMR (D_2O) δ 1.62 (s, 3 H), 1.60-2.00(m, 2 H), 3.40-4.90 (m, 8 H), 7.35 (s, 1 H).

A solution of 6 (1 mg) in aqueous NaHCO_3 (pH 10.5, 10 mL) was heated at 70 °C for 2 h. HPLC analysis revealed the formation of 7 in 90% yield.

1-(1-Thyminy1)-2-hydroxypropyonic acid (7). UV (H_2O) 272 nm ($\log \epsilon$ 3.84); ^1H NMR (D_2O) δ 1.87 (s, 3 H), 4.13 (d, 2 H, $J = 6$ Hz), 5.08 (t, 1 H, $J = 6$ Hz), 7.53 (s, 1 H); ^{13}C NMR (D_2O) δ 12.3 (q), 60.3 (t), 62.3 (d), 111.5 (s), 142.5 (d), 152.9 (s), 167.2 (s), 171.9 (s). Exact mass. Found: 242.0888. Calcd. for $\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_5$ (methylester): 242.0903.

B. With L-Asparagine. A solution of 1 (121 mg, 0.5 mmol) and L-asparagine (300 mg, 2 mmol) in distilled water (200 mL) at pH 11 was irradiated as described above for 40 h. After irradiation the solution was heated at 70 °C for 2 h and then lyophilized to dryness. Preparative HPLC (methanol-water (1:1)) of the residue gave 8 as a colorless syrup (56 mg, 30% based on consumed 1).

1-(1-Thyminy1)-2-carbamoylpropyonic acid (8). ^1H NMR (D_2O) δ 1.69 (s, 3 H), 2.78 (dd, 1 H, $J = 12, 8$ Hz), 3.05 (dd, 1 H, $J = 12, 6$ Hz), 5.03 (dd, 1 H, $J = 8, 6$ Hz), 7.31 (s, 1 H); ^{13}C NMR (D_2O) δ 12.2 (q), 36.0 (t), 59.2 (d), 111.8 (s), 143.0 (d), 152.5 (s), 167.3 (s), 172.8 (s), 175.1 (s).

C. With Glycine. A solution of 1 (24 mg, 0.1 mmol) and glycine (75 mg, 1 mmol) in distilled water (100 mL) at pH 11.0 was irradiated for 2 h under the standard conditions. After irradiation the photolysate was heated at 70 °C for 2 h. Removal of the solvent followed by preparative HPLC gave 9 as a colorless syrup (14 mg, 45% based on consumed 1).

1-(1-Thyminy)acetic acid (9). ^1H NMR (CD_3OD) δ 1.93 (s, 3 H), 4.50 (s, 2 H), 6.95 (s, 1 H). Exact mass. Found: 212.0898. Calcd. for $\text{C}_9\text{H}_{12}\text{N}_2\text{O}_4$ (methylester): 212.0797.

D. With L-Histidine. A solution of 1 (121 mg, 0.5 mmol) and L-histidine (310 mg, 2 mmol) in distilled water (200 mL) at pH 12.0 was irradiated for 10 h as described above. A similar work up gave 10 as a colorless syrup (120 mg, 30% based on consumed 1).

1-(1-Thyminy)-2-(2'-imidazolyl)propyonic acid (10). ^1H NMR (D_2O) δ 1.66 (s, 3 H), 3.00-3.70 (m, 2 H), 5.00 (m, 1 H), 7.09 (s, 1 H), 7.23 (s, 1 H), 8.46 (s, 1 H); ^{13}C NMR (D_2O) δ 12.2 (q), 26.8 (t), 61.3 (d), 111.8 (s), 117.8 (d), 130.3 (s), 134.4 (d), 141.9 (d), 152.7 (s), 167.0 (s), 174.4 (s).

Irradiation of 1 with Poly-L-Lysine. A solution of 1 (0.24 mg, 0.001 mmol) and poly-L-lysine hydrobromide (20 mg) in 0.04 M Na_2CO_3 buffer (5 mL) at pH 10.5 was irradiated with a 400 W medium-pressure mercury lamp through Vycor filter for 1 h externally. After irradiation the photolysate was heated at 90 °C for 2 h and then lyophilized to dryness. The residue was dissolved in 6 N HCl (5 mL), and the solution was heated at 110 °C in a sealed tube for 22 h. The hydrolysate was evaporated to dryness in vacuo, and the residue was dissolved in water (5 mL) and subjected to HPLC analysis. The amount of 2 with retention time of 6.5 min was determined by HPLC. Identification of 2 was made by comparison of chromatographic behaviours (TLC, HPLC) with those of authentic sample after collection of the HPLC fraction corresponding to 2.

Irradiation of 1 with Histone. A solution of 1 (0.24 mg,

0.001 mg) and Histone V-S (3 mg) in distilled water (5 mL) at pH 10.5 was irradiated for 1 h as described above. After irradiation the photolysate was heated at 90 °C for 2 h and then lyophilized to dryness. The residue was dissolved in 6 N HCl (5 mL), and the solution was heated at 110 °C in a sealed tube for 22 h. The hydrolysate was evaporated to dryness in vacuo, and the residue was dissolved in water and subjected to HPLC analysis.

Irradiation of Calf Thymus DNA with Poly-L-Lysine. At pH 10.5.
A solution of calf thymus DNA (2.7×10^{-4} M (phosphate)) and poly-L-lysine hydrobromide (2.7×10^{-4} M (amine)) in 2.5 mM Na_2CO_3 -0.25 mM EDTA buffer (200 mL). After irradiation the solution was heated at 90 °C for 2 h and then lyophilized to dryness. The residue was dissolved in 6 N HCl (10 mL), and the solution was heated at 110 °C in a sealed tube for 22 h. The hydrolysate was evaporated to dryness in vacuo, and the residue was dissolved in water and subjected to HPLC. HPLC analysis of the hydrolysate indicated the presence of 2 in 0.5% yield on the basis of thymine contained in DNA (27.6%).

At pH 7.5. A solution of calf thymus DNA (2.7×10^{-4} M) and poly-L-lysine hydrobromide (2.7×10^{-4} M) in 2.5 mM NaHCO_3 -0.25 mM EDTA buffer (200 mL). After a similar work-up HPLC analysis of the hydrolysate indicated the presence of 2 in 0.1% yield on the basis of thymine contained in DNA.

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Chapter 3

Selective Removal of Thymine from DNA by using Photoreaction with Alkylamine

ABSTRACT

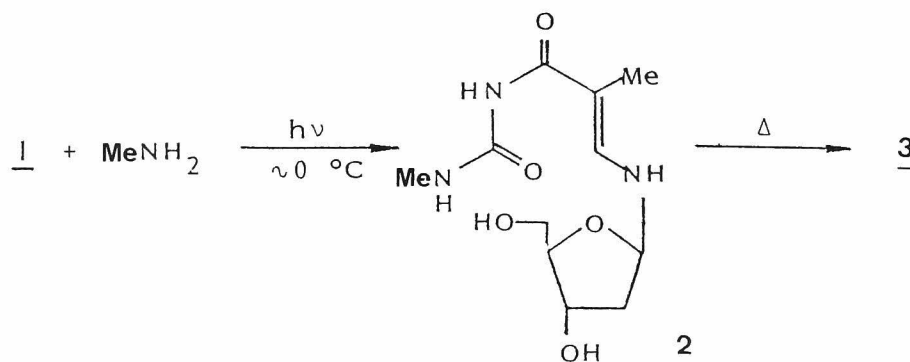
High selectivity toward Thd has been observed when the photoreaction of a mixture of dAdo, dCyd, dGuo and Thd with methylamine was carried out at 0°C in the presence of diazabicyclo[2.2.2]octane (0.2 mM) and then the photolysate was kept at 20°C after irradiation. Irradiation of calf thymus DNA and methylamine under the specified conditions followed by heating at 70°C led to the efficient release of 1-methylthymine (3) from DNA. The extent of DNA modification is readily determined by the absorbance change at 300 nm which corresponds to the amount of the ring-opened adduct between methylamine and thymine in DNA. Model experiments using thymidyl(3'-5')-2'-deoxyadenosine demonstrate that the 3'-5' phosphodiester linkage is cleaved efficiently in this photoreaction. The present results indicated the irradiation of DNA with methylamine induces a exceedingly facile removal of thymine from DNA with the strand cleavage at the reacting thymine, providing a convenient and potentially useful method for thymine selective modification of DNA.

INTRODUCTION

Chemical modification of nucleic acid components is one of the promising approaches for studying the structure and function of nucleic acids.¹ The developments of base specific chemical reactions of nucleic acid constituents is extremely important for modification of nucleic acids. The most prominent example so far known for the use of base specific chemical reactions is Maxam-Gilbert method for sequencing DNA.² In our study to explore the chemical basis of UV induced nucleic acid-protein crosslinks,³ we have found that photo-excited thymidine reacts with primary amines to produce a ring-opened adduct which on subsequent heating is readily converted to N(1)-substituted thymine in high yields.⁴ We have felt that this novel type of photochemical conversion would be used for a specific modification of thymine moieties in DNA. In the present chapter we describe the details of the photochemistry of thymidine in the presence of amines and its application to selective removal of thymine from DNA.⁵

RESULTS AND DISCUSSION

Selectivity of the Photoreaction. The conversion of thymidine (1, Thd) to ring-opened adduct 2 proceeds in high yield on irradiation with methylamine at low temperature and represents a key part of our method for the thymine selective modification of DNA, while the isolated yield of 2 is not so high because of its thermal instability.



When Thd and methylamine were irradiated at 0 °C in 0.1 M NaHCO₃ aqueous solution, a new absorption band of 2 appeared around 300 nm quantitatively. Under the same conditions, irradiation of 2'-deoxyadenosine (dAdo) and methylamine did not give any detectable product, and dAdo was recovered quantitatively. Irradiation of 2'-deoxycytidine (dCyd) and methylamine in NaHCO₃ aqueous solution at 0 °C resulted in the formation of unstable dCyd photohydrate as reported previously.⁶ However, on warming the photolysate to 20 °C dCyd reverted rapidly to dCyd in more than 95% yield as determined by HPLC.^{6e,7} Under the irradiation conditions, deamination of dCyd to 2'-deoxyuridine (dUrd) has not been observed. Photoreaction between 2'-deoxyguanosine (dGuo) and methylamine under nitrogen atmosphere resulted in a considerable decrease of dGuo. Since dGuo is known to be susceptible to photooxidation,⁸ disappearance of dGuo is probably due to the oxidation with trace of oxygen present in the reaction system. In fact, addition of diazabicyclo[2.2.0]octane (Dabco), a singlet oxygen quencher, to the reaction system considerably inhibited the disappearance rate for Thd vs. dGuo is ca. 5 : 1 under the irradiation conditions (Figure 2). Thus, relatively high selectivity toward Thd has been observed when photoreaction of the DNA components with methylamine was conducted at 0 °C in aqueous NaHCO₃ solution containing 0.2 mM of Dabco under nitrogen atmosphere and the photolysate was then allowed to warm to 20 °C. Under the regulated conditions, irradiation of a mixture of four DNA components (dAdo, dCyd, dGuo and Thd) in the presence of methylamine resulted in a preferential reaction with Thd as illustrated in Figure 2. It seems probable that much higher selectivity toward thymine would be expected in the photoreaction of polynucleotides such as DNA in the presence of methylamine as in the case of many other chemical modifications of polynucleotides.

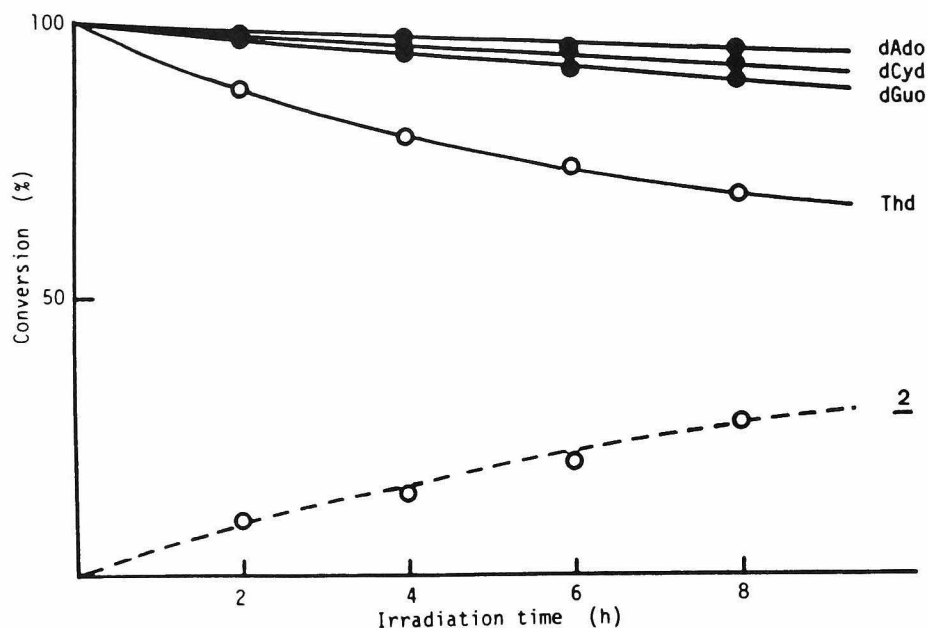


Figure 1. Photoreaction of a mixture of nucleosides (dAdo, dGuo, dCyd and Thd) in the presence of methylamine at pH 10.5. After irradiation at 0 °C, the solution was kept at 20 °C for 20 min, and the disappearance of nucleotides (—) and the formation of 2 (---) were determined by HPLC and UV spectroscopy, respectively.

Photoreaction of DNA with methylamine. We next examined the photoreaction of DNA with methylamine. When heat-denatured calf thymus DNA in 0.1 M NaHCO₃ aqueous solution (pH 10.5) containing methylamine and Dabco was irradiated at 0 °C under the specified conditions and the progress of the reaction was monitored with UV spectroscopy at room temperature, a new absorption band around 300 nm was increased with increasing irradiation time with the isosbestic points at 245 and 289 nm, in exactly the same manner as in the case of photoreaction between four DNA components and methylamine (Figure 2). The photolysate was then heated to 70 °C for 2 h. Upon this treatment the absorption at 300 nm mainly due to the ring-opened product of thymine (7) was decreased with concomitant formation of 1-methylthymine (3) as evidenced by HPLC analysis. The amount of

reacted thymine in DNA is readily calculated from the absorbance change at 300 nm (ΔAbs_{300}) before and after heating the photolysate at 70 °C, by assuming that the ring-opened adduct of thymine in DNA⁹ has the same molar extinction coefficient as that of 2 ($\epsilon = 14,800$ at 300 nm). 1-Methylthymine (3) thus released from DNA was determined directly by HPLC without any systematic degradations of DNA. As shown in Table I, external irradiation with a 10 W low pressure mercury lamp through Vycor filter for 6 h resulted in the release of 3 in 10% yield based on thymine contained in DNA.¹⁰

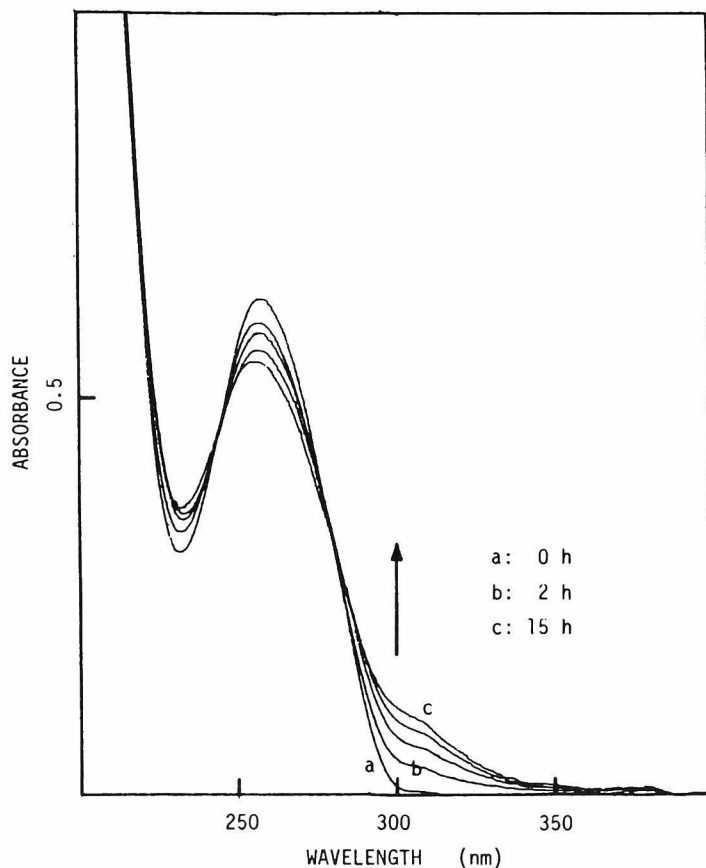


Figure 2. Progressive UV spectral change induced by irradiation of heat-denatured calf thymus DNA in 0.1 M aqueous NaHCO_3 at pH 10.5 in the presence of methylamine at 0 °C. In each run, UV absorption spectrum was measured after photolysate was kept at 20 °C for 20 min.

Table I. Variation of Product Yield with Irradiation Time in the Photoreaction of DNA in the Presence of Methylamine^a

irradiation time, h	ΔAbs_{300}^b	calculated yield of <u>7</u> in DNA, ^c %	yield of released <u>3</u> , ^d %
2	0.025	7.5	3.9
4	0.045	13.5	7.4
6	0.057	17.1	10.1
8	0.068	20.4	13.3
15	0.072	21.6	15.3

^a Reaction conditions are the same as indicated in Figure 2.

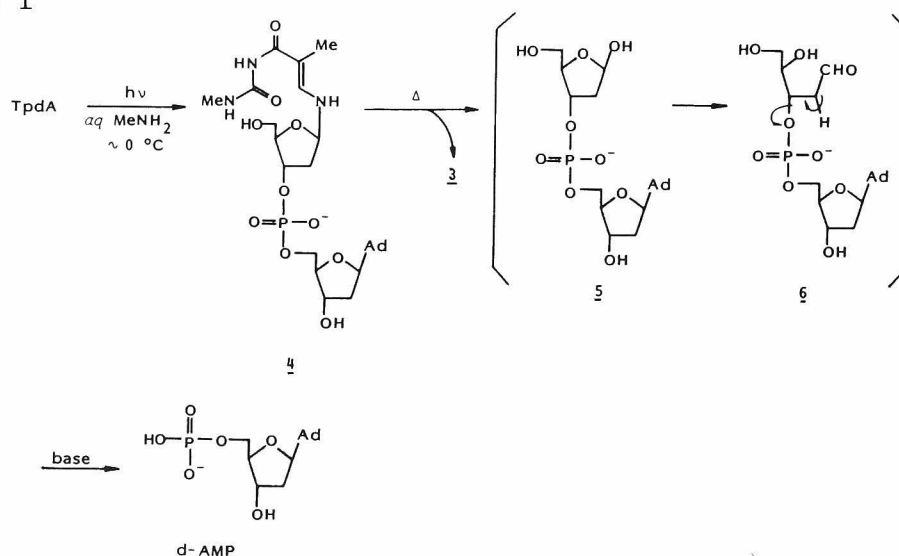
Yields are based on thymine contained in DNA (27.6%, $8.34 \times 10^{-5}\text{M}$).

^b Absorbance change at 300 nm before and after heating the photolysate at 70 °C. ^c The yield was calculated from the absorbance change (ΔAbs_{300}). ^d Yield determined by HPLC.

In order to ascertain whether the phosphodiester linkage of the DNA chain is cleaved in this photoreaction, we have carried out the photoreaction of a dinucleotide as a simple model. When thymidyl- (3'-5')-2'-deoxyadenosine (TpDA) and methylamine were irradiated for 15 min at 0 °C, a new UV absorption due to the ring-opened adduct 4 appeared at *ca* 300 nm in a quantitative yield as estimated by the absorbance change (ΔAbs_{300}). After being heated at 70 °C for 2 h, the photolysate was analyzed by HPLC to reveal the presence of 8% of 3, 12% of 2'-deoxyadenosine-5'-monophosphate (d-AMP) and 88% of unreacted TpDA. Irradiation for 45 min and successive heating produced 3, d-AMP and TpDA in 15, 24, and 68% yields, respectively. These results clearly indicate that the 3'-5' phosphodiester linkage of TpDA is efficiently cleaved giving rise to 3 and d-AMP by irradiation and subsequent heating in alkaline aqueous solution. The formation of 3 and d-AMP may be reasonably explained by the mechanism shown in Scheme

I. The ring-opened adduct 4 formed by the reaction with methylamine would undergo intramolecular cyclization and β -elimination to give 3 and 5 as mentioned earlier. The cleavage of phosphodiester linkage would proceed via unmasked sugar aldehyde 6 by a well known base-catalyzed β -elimination mechanism.⁹ Thus the present experiment suggests that irradiation of DNA with methylamine and subsequent heating of the photolysate may induce strand scission at the reacting thymine.

Scheme I



Chemical reactions resulting in cleavage of certain base sequences of polynucleotides are of particular importance in investigating the biological function of nucleic acids and the mechanism of action of mutagens, carcinogens¹¹ and anticancer agents.¹² For example, anticancer agent, neocarzinostatin, has been demonstrated to induce DNA strand breaks with liberation of thymine from DNA.^{12,13} In the present work we have demonstrated that irradiation of DNA with methylamine followed by heating at 70°C results in an exceedingly facile removal of thymine from DNA. Model experiments using dinucleotide TpdA suggest that the photoreaction of DNA components strongly induces strand scission at the reacting thymine as illustrated in Scheme II. Observed high selectivity toward Thd in the photoreaction of DNA components strongly suggests that thymine is preferentially

Table II. Product Distribution in the Photoreaction of TpdA in the Presence of Methylamine^a

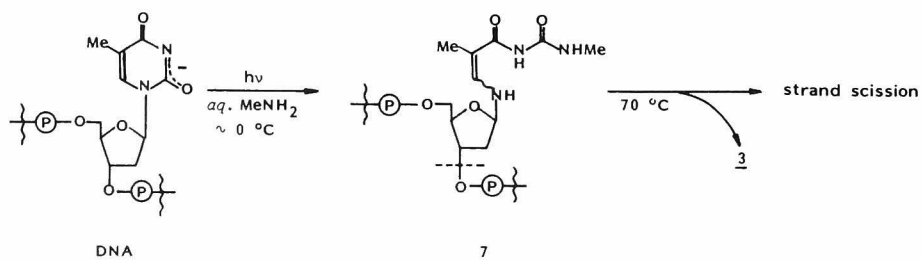
irradiation time, min	TpdA, ^b %	product, ^b %	
		<u>3</u>	d-AMP
15	88	8	12
30	78	13	18
45	68	15	24

^a Reaction conditions are indicated in Experimental Section.

^b Yield determined by HPLC.

attacked by methylamine in the irradiation of DNA, although the selectivity in the photoreaction of DNA itself is the subject of further investigation. Owing to its simplicity and the easiness in monitoring reaction, the present photoreaction may provide a convenient and potentially useful method for thymine selective modification of DNA. We also observed that a similar type of photoreaction can take place between DNA and lysine residues of histones in DNA-histone complexes, which is the subject of chapter 5.

Scheme II



EXPERIMENTAL

Ultraviolet spectra were recorded with a Shimadzu UV-200 spectrophotometer. High-performance liquid chromatography (HPLC) was performed on a Waters ALC/GPC 204 model equipped with a 254-nm or a 280-nm fixed-wavelength detector. Reverse-phase μ Bondapak C₁₈ and Nucleosil 7C₁₈ (1 x 25 cm) columns were used for analytical and preparative HPLC, respectively. TLC was performed on a silica gel plate (Merck 60 PF₂₅₄).

Irradiations were carried out by using a 10-W low-pressure mercury lamp (method A) or a 400-W high-pressure mercury lamp (method B). Vycor filters were used to cut off wavelengths $\lambda < 240$ nm in all cases.

Calf thymus DNA was purchased from P-L Biochemicals and used without purification. dAdo, dGuo, dCyd, Thd, d-AMP, and TpdA were obtained from Sigma.

Selectivity of the Photoreaction. A solution of dAdo (0.2 mM), 40% methylamine (1 mL, 10 mM) and Dabco (0.2 mM) in 50 mM aqueous NaHCO₃ at pH 10.5 was irradiated at 0 °C by method A for 100 min. HPLC analysis (acetonitril-water-acetic acid (40 : 60 : 1)) of the mixture at room temperature indicated no loss of dAdo.

A solution of dCyd (0.2 mM) and methylamine (20 mM) in 50 mM NaHCO₃ was irradiated at 0 °C under the same conditions for 30 min. Progress of reaction was monitored by UV spectroscopy and HPLC. The UV spectrum of the photolysate immediately after irradiation showed the disappearance of dCyd absorption (270 nm). HPLC analysis indicated the presence of a new peak ascribable to dCyd photohydrate⁶ with retention time of 6 min in addition to unreacted dCyd. When the photolysate was kept at 20 °C for 20 min, the peak of the photohydrate completely disappeared with the enhancement of the dCyd peak. The reversibility is more than 95% as determined by HPLC.

When a solution of dGuo (0.2 mM) and methylamine (20 mM) was irradiated at 0 °C under nitrogen atmosphere for 30 min, 10% of the dGuo was consumed, as determined by HPLC. However, the same irradiation in the presence of Dabco (0.2 mM) resulted in 96% recovery of dGuo.

A solution containing dAdo (0.70 mM), dGuo (0.58 mM), dCyd (0.54 mM), and Thd (0.69 mM) was irradiated at 0 °C by method A in the presence of methylamine (0.1 M) and Dabco (0.2 mM) in 0.1 M aqueous NaHCO₃ at pH 10.5 under nitrogen atmosphere. After irradiation, the solution was kept at 20 °C for 20 min, and the disappearance of nucleosides and the formation of 2 were determined by HPLC (acetonitrile-water-acetic acid (40 : 60 : 1)) and UV spectroscopy, respectively. The results are shown in Figure 1.

Irradiation of Calf Thymus DNA in the Presence of Methylamine.

A solution of heat-denatured calf thymus DNA (50 mg), 40% methylamine (1 mL, 10 mmol), and Dabco (2.2 mg, 0.02 mmol) in 0.1 M aqueous NaHCO₃ (100 mL) at pH 10.5 was irradiated externally with a 10-W low-pressure mercury lamp through a Vycor filter at 0 °C under nitrogen atmosphere. Equivalent volumes (5 mL) of the photolysate were withdrawn periodically, and UV absorption spectra were measured after the solution was kept at 20 °C for 20 min. The solutions were then heated at 70 °C for 2 h, and their UV spectra were recorded again. Absorbance changes at 300 nm (ΔAbs_{300}) before and after heating at 70 °C were calculated in each case. After being passed through a membrane filter (Millipore FH, 0.5 μm) to remove DNA, the sample solutions were subjected to HPLC analyses (methanol-water (1 : 9), 2 mL/min). The amounts of 3 with retention time of 5 min were determined by using a Waters Data Module 730. Identification of 3 was made by comparison of chromatographic behaviors (TLC, HPLC) and UV spectrum with those of authentic sample after collection of the HPLC peak. The results are shown in Figure 2 and Table I.

Irradiation of Thymidylyl(3'-5')-2'-deoxyadenosine (TpdA) and Methylamine. A solution of TpdA (0.353 mg, 59 μmol) and 40% methylamine (10 μl) in distilled water (10 mL) at pH 11.8 was irradiated internally by method B at 0 °C. Progress of reaction was monitored by the appearance of 300-nm absorption. After irradiation, aliquots of the photolysate were withdrawn and heated at 70 °C for 2 h. The solutions were analyzed by HPLC under the following conditions: d-AMP

(retention time 3 min; μ Bondapak C₁₈ column; methanol-0.01 M KH₂PO₄ (1 : 9), 2 mL/min); 1-methylthymine (3) (retention time 5 min; μ Bondapak C₁₈ column; methanol-water (1 : 9), 2 mL/min). The results are shown in Table II.

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CHAPTER 4

A New Procedure for Determining Thymine Residues in DNA Sequencing. Photoinduced Cleavage of DNA Fragments in the Presence of Spermine

ABSTRACT

A new procedure for T specific cleavage of DNA fragments utilizing photoreaction with spermine has been described. Irradiation of 3'-[^{32}P]-end-labeled DNA fragments for 10 - 20 min with a germicidal lamp emitting mainly 254-nm light in the presence of 1 M spermine in distilled water resulted in a T specific cleavage of the DNA chains. This method does not require piperidine treatment. By contrast, when the DNA fragments were irradiated in the presence of methylamine under similar conditions, both G and T bands with the intensity of $G > T$ have appeared. A similar but less selective T cleavage has also been observed in the irradiation of 5'-[^{32}P]-end-labeled DNA fragments in the presence of spermine followed by brief heating of the photolysate in a loading buffer for gel electrophoresis. The T specific photoreaction with spermine and the $G > T$ reaction with methylamine described here may be conveniently used in combination with the standard Maxam-Gilbert's reactions to provide independent confirmatory readings.

INTRODUCTION

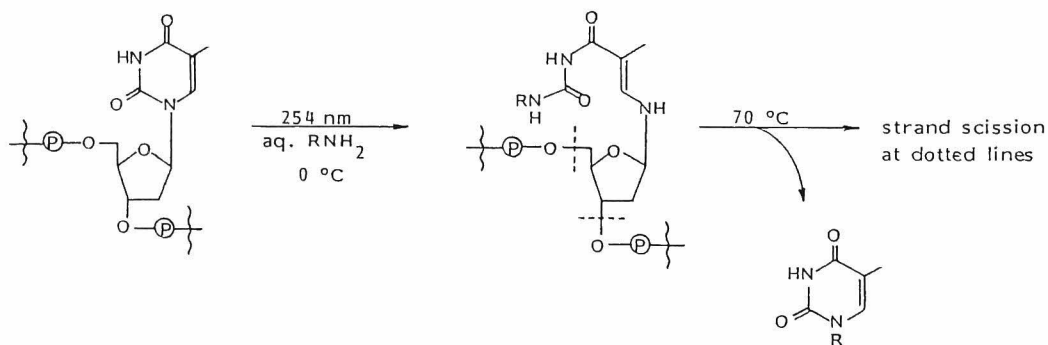
While the Maxam-Gilbert method based on base selective chemical reactions of end-labeled DNA has been widely used for sequencing DNA,¹ there still needs a simple and reliable method for T specific cleavage of DNA. The pyrimidine specific reactions using hydrazine in the Maxam-Gilbert method are somewhat less reliable than those used for purines, and their specificity is sensitive to reaction conditions. Occasionally, incomplete suppression of T reaction by adding NaCl or weakened T cleavage in the C + T reaction occurs in the hydrazine reactions, and this may cause the difficulty to distinguish C and T in chemical DNA sequencing.^{2,3,4} To attain a T specific reaction, oxidative modifications using potassium permanganate⁴ and osmium tetroxide² have recently been reported. While these methods lead to a T selective cleavage of DNA fragments, the variability in band strength at T residues has also been reported in both cases.^{2,4} In this chapter, we report a new, convenient method for T specific cleavage of DNA fragments by utilizing a T selective photoreaction with primary amines recently discovered in our laboratory.^{5,6} Our method is easy to perform without hazardous reagents and gives bands of our method is to eliminate the step of time-consuming piperidine treatment.

RESULTS AND DISCUSSION

In the preceding chapter we have demonstrated that irradiation of a dinucleotide, *e.g.*, thymidyl-(3'-5')-2'-deoxyadenosine, in the presence of methylamine produces a ring-opened adduct of the pyrimidine ring which on subsequent heating at 70°C leads to an efficient cleavage of the 3'-5' phosphodiester linkage via β -elimination.⁶ We viewed that the sequence of these reactions may be used for T specific cleavage of DNA fragments. While our work was in progress, Simoncsits and Török reported that irradiation of 5'-[³²P]-labeled DNA fragments in the presence of primary alkylamines followed by

piperidine treatment (90 °C, 30 min) led to the cleavage of the DNA chains at T residues, being accompanied by a less intensive G cleavage together with faint C and A reactions.¹⁰ Our previous work has suggested that the G cleavage is due to the photooxidation of G residues which can be suppressed by addition of a singlet oxygen quencher,⁶ whereas the minor C cleavage is assumed to result from the alkaline-sensitive lesions at pyrimidine-cytosine sequences recently observed by Haseltine and co-workers in 254-nm irradiation of human DNA.^{11,12} We also know from the model experiments that the T cleavage induced by the photoreaction with primary alkylamines does not require piperidine treatment.⁶ Based on these considerations, we devised a new method for highly T selective cleavage of DNA fragments by utilizing a photoreaction with spermine which is known to strongly bind to DNA and may act as an inhibitor for G photooxidation.

Scheme 1



Figures 1 and 2 show the autoradiographs of the sequencing gels of UV-irradiated two 3'-[³²P]-end-labeled DNA fragments of defined sequences together with the standard sequencing reactions of the Maxam-Gilbert method. Irradiation for 10 - 20 min with a commercial germicidal lamp emitting mainly 254-nm light in the presence of 1 M spermine in distilled water resulted in a T specific cleavage of the DNA chain of short (56 bp) and long (178 bp) DNA fragments without piperidine treatment. The longer the irradiation time the more intensive bands at T residues appeared as seen in Figure 2. It is noteworthy that only the T bands appeared uniformly within 20 min

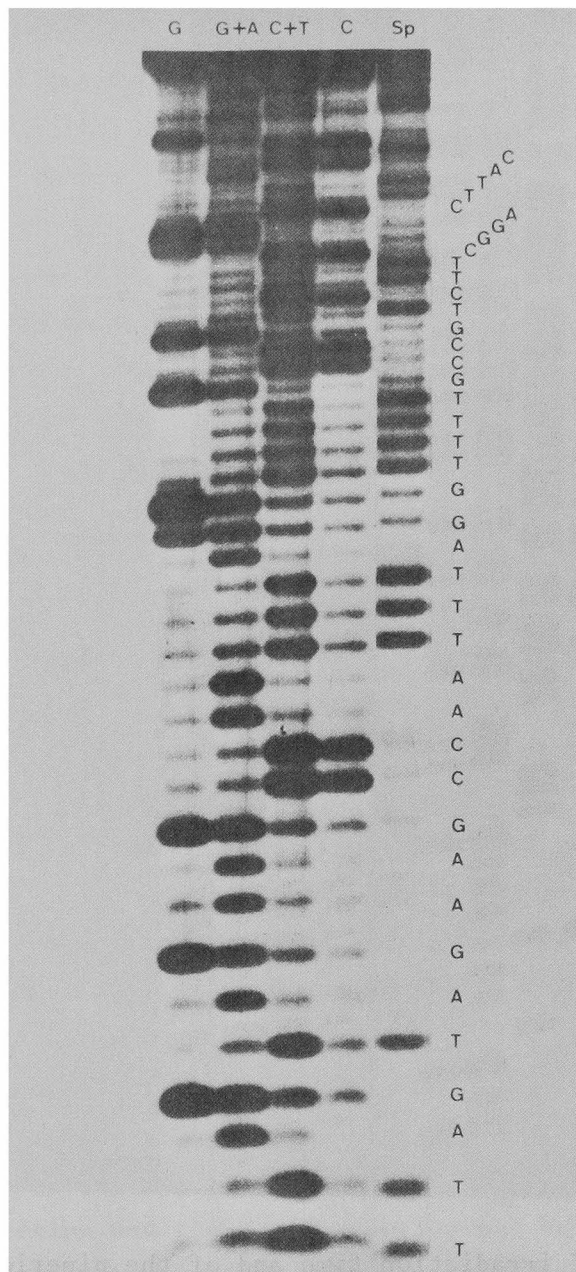


Figure 1. Autoradiograph of sequencing gel of short DNA fragment (56 bp) showing both the Maxam-Gilbert reactions (G, G + A, C + T, C) and the spermine photoreaction (Sp).

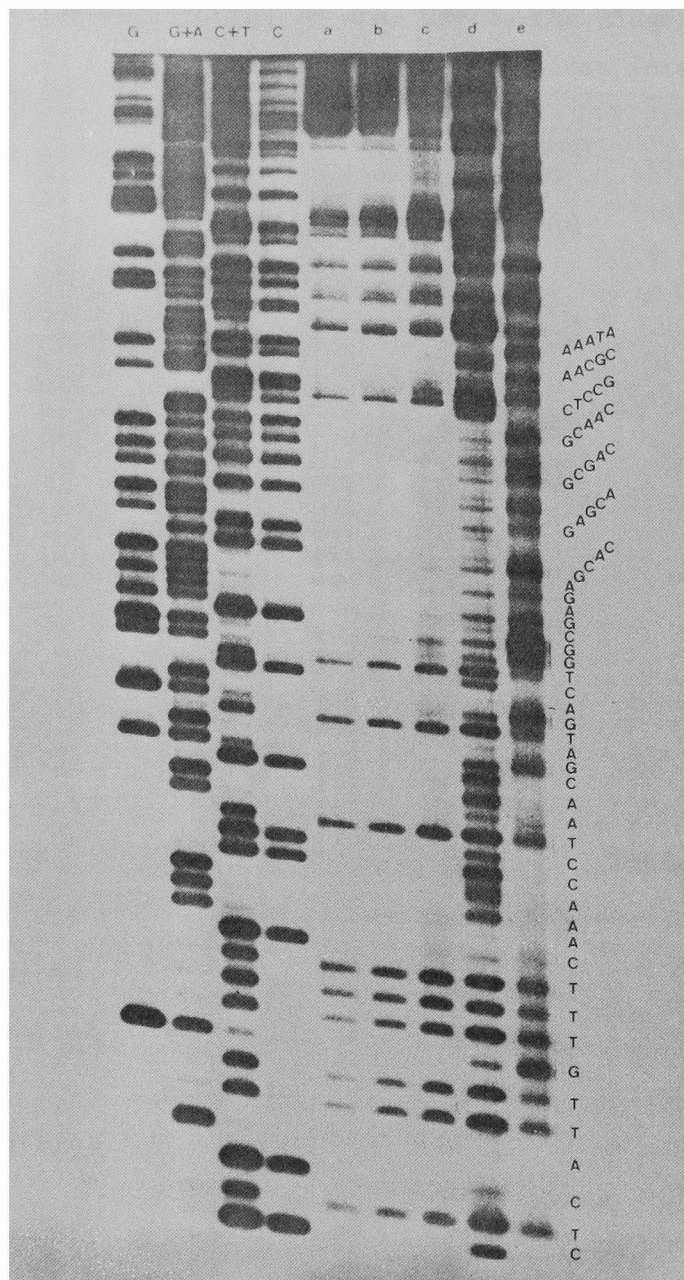


Figure 2. Effect of irradiation time and of the piperidine treatment on the photoreaction of a long DNA fragment (178 bp) with spermine. In lane a, b and c, the irradiation was performed for 10, 20 and 30 min, respectively. In lane e, irradiation was performed in the presence of methylamine for 20 min.

irradiation, whereas prolonged irradiation (> 30 min) resulted in minor side-reactions together with intense T bands (Figure 2, lane c). The band pattern was not altered significantly, when the irradiation was performed under nitrogen atmosphere. However, when the same irradiation was followed by treatment with 1 M piperidine (90 °C, 30 min), the strong bands at T residues were accompanied with less intensive bands at G, C and A residues (Figure 2, lane d). When the DNA fragment was irradiated in the presence of methylamine in place of spermine under similar conditions, both G and T band with the intensity of G > T have appeared without piperidine treatment (Figure 2, lane e). Other primary alkylamines such as *n*-butylamine and cyclohexylamine^{5,6} gave essentially the same result. Unlike other alkylamines, spermine retards the G reaction presumably by chemical or physical quenching of singlet oxygen generated under the irradiation conditions.^{6,13}

With a single exposure to UV light for 20 min, we have been able to identify T residues in sequences up to 100 base pairs in length. A similar T specific cleavage has also been observed in the irradiation of a 5'-[³²P]-end-labeled DNA fragment with spermine. In this case, however, a prolonged heating (90 °C, 10 min) of the irradiated DNA fragment in a loading buffer for gel electrophoresis is necessary to attain a T specific reaction. Treatment of the irradiated 5'-end-labeled fragment with piperidine (90 °C, 30 min) resulted in T + G cleavage together with minor C reactions as already reported.¹⁰

The T specific reaction with spermine and the G > T reaction with methylamine described above gave reproducible results on several short and long 3'-[³²P]-end-labeled DNA fragments. The present T specific reaction and the G > T reaction may be conveniently used in combination with the standard Maxam-Gilbert's C + T reaction to provide independent confirmatory readings. Furthermore, the present observations suggest that a similar type of photoinduced cleavage of DNA chain with primary amines may play an important role in UV-induced damage on cells.

EXPERIMENTAL

Restriction enzymes *Hae*III, *Tag*I and *Hinf*I, and T4 polynucleotide kinase were obtained from Takara Shuzo Co. Ltd., the klenow fragment of DNA polymerase I of *Escherichia coli* was from Bethesda Research Laboratories GmbH, and calf intestine alkaline phosphatase was from Boeringer Mannheim GmbH. [α - 32 P]dCTP and [γ - 32 P]ATP (specific activity of about 3000 Ci/mmol) were purchased from New England Nuclear a Du Pont Co., and Amersham International plc. Commercially available spermine and methylamine were used without purification as 2 M stock solutions in distilled water. Irradiation was made with a commercial 15 W germicidal lamp (Toshiba GL 15).

Three DNA fragments of defined sequences were obtained from bacteriophage ϕ X174 replicative form DNA. Double-stranded ϕ X174 replicative form DNA was prepared as previously described⁷ and digested with *Hae*III, and 194 and 234 base pair fragments [Z_8 and Z_7 fragments in the map reported by Sanger⁸] were purified. Fragment Z_7 was digested with *Taq*I and labeled by extension of the 3' termini with Klenow polymerase in the presence of [α - 32 P]dCTP.⁹ Resulting 3'-end labeled 56 and 178 base pair fragments (C_{436} - C_{491} and C_{492} - C_{669} in the map reported by Sanger, respectively) were purified by electrophoresis on a 6% polyacrylamide gel. 5'-end labeled DNA fragments were obtained by incubation of the Z_7 or Z_8 fragment with [γ - 32 P]ATP and T4 polynucleotide kinase. After digestion with *Taq*I or *Hinf*I, 5'-end labeled 55 and 139 base pair fragments (C_{436} - T_{490} , C_{980} - G_{1118}) were purified by electrophoresis on a 6% polyacrylamide gel.

10 μ L of the 3'-end-labeled DNA fragment was mixed with 10 μ L of 2 M spermine in a 1.5 mL Eppendorf tube. The open sample tube was cooled with ice-water at 0 °C and exposed to UV light at a distance of 25 cm for 10 - 20 min. Then 230 μ L of 0.3 M sodium acetate buffer containing 0.1 mM EDTA and 25 μ g/mL of tRNA and 750 μ L of 95% ethanol were added to the solution at 0 °C. The mixture was chilled for 10 min at -70 °C and centrifuged at 12,000 g for 10 min. The pellet

was resuspended in 250 μ L of 0.3 M sodium acetate buffer (pH 5.2), and DNA was precipitated with ethanol again. The pellet was rinsed in 70% ethanol, dried and dissolved in 5 μ L of 80% formamide-10 mM NaOH loading buffer for gel electrophoresis. The solution was heated for 1 min at 90 °C and loaded on 10% or 16% polyacrylamide sequencing gel. In the case of the 5'-end-labeled DNA fragment, the pellet was dissolved in 5 μ L of 80% formamide-10 mM NaOH loading buffer, heated at 90 °C for 10 min and loaded on polyacrylamide sequencing gel.

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CHAPTER 5

Isolation and Characterization of a Thymine-Lysine Adduct in UV-irradiation Nuclei. The Role of Thymine-Lysine Photoaddition in Photo-Cross-Linking of Proteins to DNA

ABSTRACT

In order to know the feasibility of thymine-lysine photoaddition in chromatin, irradiations of calf thymus nucleohistone and of chicken erythrocyte nuclei were examined in NaHCO_3 - Na_2CO_3 buffer. After irradiation, the photolysates were heated at 70 °C and then subjected to acid hydrolysis (6 N HCl, 110 °C). Analysis of the hydrolysates by HPLC and amino acid analyzer revealed the presence of 2 in both cases. Ultimately, 2 was isolated by preparative HPLC and identified by comparison of the 400-MHz ^1H NMR with that of the authentic sample. We also observed that free histones were released from UV crosslinked histone-DNA adducts after heating at 90 °C for 2 h. These observations indicated that the photoexcited state of thymine in DNA reacts with neighboring lysine ϵ -amino groups of histones to result in the formation of DNA-histone adducts which upon subsequent heating release histones containing partially modified lysine residues. The chemical mechanism for the photo-cross-linking of histones to DNA in core nucleosome has been proposed.

INTRODUCTION

DNA-protein crosslinks are formed when cells are exposed to γ - or UV-irradiation. These linkages may be important in cell killing, mutagenesis, and carcinogenesis, and investigation of this type of cross-linking is particularly useful in understanding the nature of radiation-induced damage in cells.¹ In eukaryotes, chromosomal DNA is packed tightly into small subunits called nucleosomes. Its structure is based upon a complex of eight histones (two each of H2A, H2B, H3, and H4), which are tightly bound to each other, forming a globular core. On the outside of this histone core are firmly bound about 144 base pairs of DNA arranged in tight superhelical coils. Model of the histone core is shown in Figure 1.^{5d} An additional histone, H1, is associated more loosely with chromatin. A variety of interactions occur among the components of the nucleosome, and it is apparent that DNA-protein interactions play an important role in the structure and function of chromatin. Many reports has already dealt with the interaction between them by using chemical and physical methods.^{5a}

Recently several investigators has irradiated chromatin with UV light as a probe to study histone DNA interaction.^{2,3} Direct photocrosslinking technique, without the aid of other reagents, also provided an important method for studying protein-nucleic acid interaction. Photochemists are exploring the structures and mechanisms of formation of adducts of nucleic acids bases and related compounds with amino acids. These study may have direct relevance to the goal of understanding of the chemical bases of UV induced crosslinking in nucleic acid-protein complexes. However our knowledge of the actual chemical nature of adducts formed in UV-irradiated DNA-histone complex is scant.^{2c,4}

The positive charge on the side chains of lysine and arginine at physiological pH are indicative that they are involved in the binding of proteins to the negative phosphates in the backbone of DNA.⁵ The potential proximity of these residues to the bases in DNA make these

amino acid of considerable interest as participants in UV induced nucleic acid-protein cross-linking. We previously noted⁶ that UV-irradiation of thymidine in the presence of primary amines, including L-lysine, produces a ring opened adduct which on subsequent heating is readily converted to N(1)-substituted thymine in high yield as outlined in Eq. 1. This type of photoreaction was successfully applied to selective removal of thymine from DNA. We also demonstrated that irradiation of calf thymus DNA in the presence of L-lysine gives 2 with concomitant cleavage of DNA strand at the reacting thymines. In this chapter we report the results demonstrating that one major cross-linking induced by UV irradiation of nuclei is the addition of ϵ -amino groups of lysine in histones to the C-2 carbon of thymine in DNA, by characterizing thymine-lysine photoadduct formed in UV irradiation of DNA-histone complexes. We also disclose that heating of DNA-histone photoadducts up to 70°C leads to the release of modified free histones which contain thyminylyl group on the ϵ -amino groups on the lysine portions.

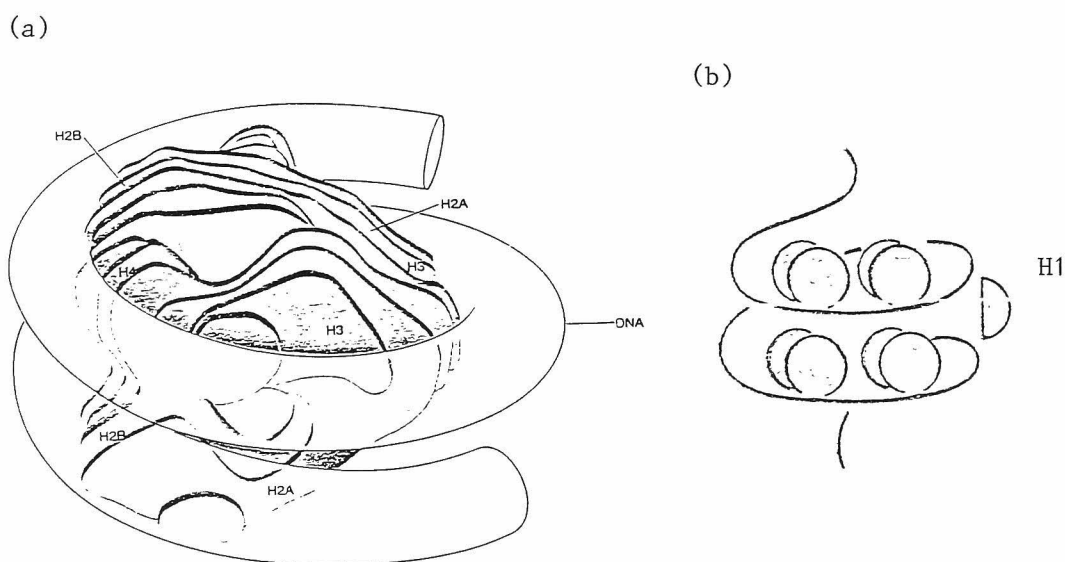
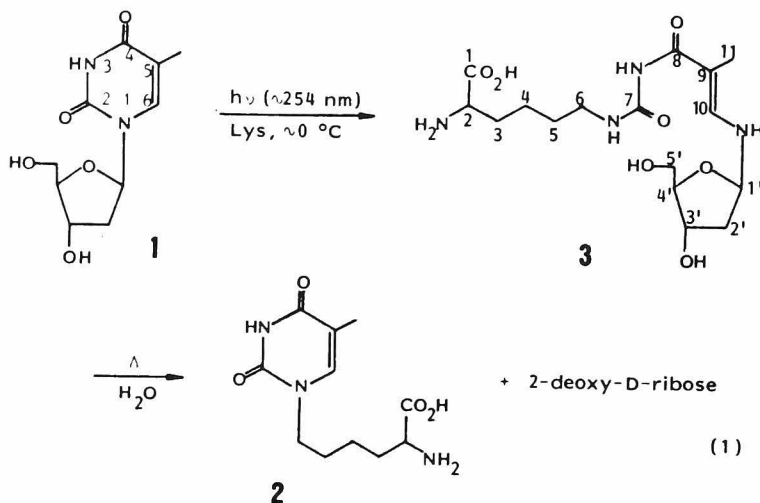


Figure 1. (a) Model of histone octamer. 144 base pairs of DNA bind on the outside of octamer.^{5d} (b) Simplified model of nucleosome.



Eq-1

(1)

RESULTS AND DISCUSSION

With information of the results of model experiments in hand we have examined the DNA-histone crosslinks induced by UV irradiation of nucleohistone. External irradiation of calf thymus nucleohistone (50 mg/200 mL) in 5 mM NaHCO_3 - Na_2CO_3 buffer (pH 10.5) was performed with a 400 W medium-pressure mercury lamp through Vycor filter ($>250 \text{ nm}$) at 10°C under nitrogen atmosphere. Following irradiation, the solution was heated at 70°C for 2 h in order to complete the conversion of **3** to **2** and then lyophilized to dryness. The sample was subjected to extensive acid hydrolysis (6 N HCl, 110°C). Analysis of the hydrolysate by HPLC or by amino acid analyzer revealed the presence of thymine-lysine adduct **2**. The modified lysine **2** appeared between methionine and isoleucine in the output of the amino acid analyzer (Figure 1).

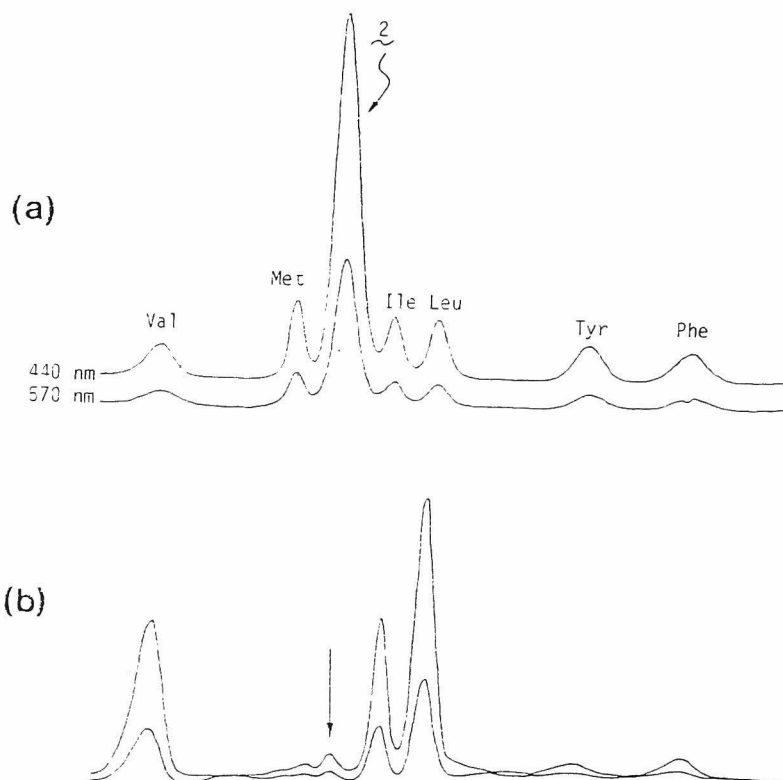


Figure 2. Output from amino acid analyzer for (a) 2 and standard mixture of amino acids, (b) the hydrolysate of irradiated nucleohistone.

Collection of the HPLC peak corresponding to 2 gave a pure product whose chromatographic behaviors, and UV spectrum are identical with those of the authentic sample obtained from the model experiment. Ultimately, the structure of the product was confirmed by direct comparison of the 400-MHz ^1H NMR with that of the authentic sample. The contents of thymine-lysine adduct 2 were directly determined by HPLC analysis of the acid hydrolysates of UV-irradiated nucleohistone under various conditions. As apparent from Table 1, the amount of 2 formed by UV irradiation of nucleohistone at pH 10.5 increased proportionally with increasing irradiation time within 40 min, and further irradiation no more increased the yield of 2 significantly. This may reflect that the lysine ϵ -amino group of intimate associa-

tion with thymine in helical DNA is primarily responsible for the formation of 2 in UV irradiation of nucleosome. In fact, addition of NaCl (up to 2 M) to the reaction system considerably retarded the formation of 2 as compared to the control experiment. Irradiation

Table 1. Contents of Thymine-Lysine Adduct 2 Determined by HPLC Analysis of UV-Irradiated Nucleohistone under Various Conditions^a

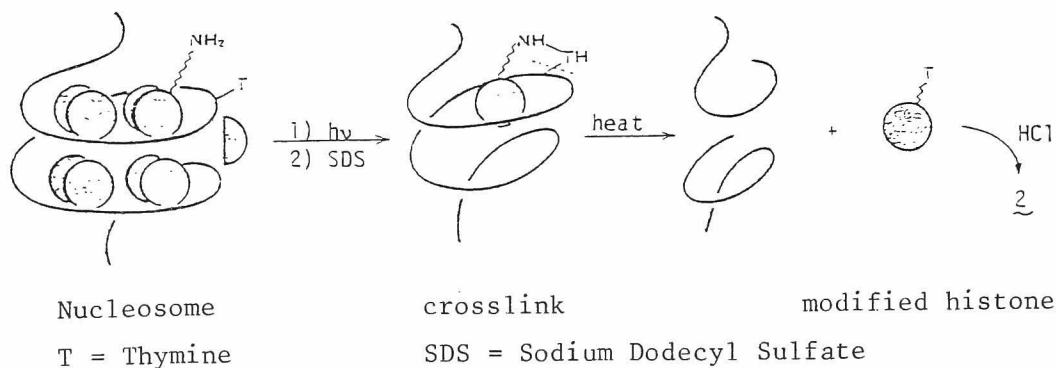
pH	irradiation time min	concn of <u>2</u> μM	yield of <u>2</u> ^b %
10.5	20	10	0.33
	40	18	0.60
	60	18	0.60
	210	20	0.67
8.6	210	5	0.17
7.5	210	4	0.13

^aSolution each containing calf thymus nucleohistone (50 mg/200 mL) in 5 mM NaHCO₃-Na₂CO₃ buffer were irradiated with a 400 W medium-pressure mercury lamp through Vycor filter at 10°C under identical conditions. Irradiated solutions were adjusted to pH 10.5, heated at 70°C for 2 h, and then lyophilized. After hydrolysis (6 N HCl), each samples were dissolved in 5 mL of water and analyzed by HPLC. ^bCalculated yields based on thymine in DNA (thymine content 27.6%) contained in calf thymus nucleohistone (DNA content 40 wt%).

at pH 7.5 proceeded less efficiently and the yield of 2 increased with increasing pH in alkaline pH region (pH 7.5 - 11) in a similar manner as observed in the photoreaction of thymidine with alkylamines as noted previously.

In order to confirm whether or not a similar photocrosslinking is feasible in irradiation of nuclei, we examined the irradiation of chicken erythrocyte nuclei (0.1 mg/mL) at 0 °C in the same buffer at pH 9.5. After being heated at 70 °C, the photolysate was hydrolyzed (6 N HCl, 110 °C) and then analyzed by an amino analyzer to reveal again the presence of 2 in the hydrolysate (Figure 3). Thus, we are able to confirm that irradiation of DNA-histone complexes in calf thymus nucleohistone and chicken erythrocyte nuclei with 254-nm light followed by extensive acid hydrolysis leads ultimately to the formation of 2. Control experiments without irradiation or irradiation with light through Pyrex filter (>280 nm) never produced 2 under the conditions. Obviously, these observations indicate that the photo-excited state of thymine N(3)-monoanion in DNA reacts with neighboring lysine ϵ -amino groups in histones to result in the formation of DNA-histone adducts which upon subsequent heating release free histone containing partially modified lysine residues. We previously demonstrated that irradiation of DNA with alkylamines in an aqueous system and subsequent heating of the photolysate at alkaline pH induce DNA strand scission at the sites of reacting thymine.⁹ It is therefore highly probable that DNA in UV-irradiated chromatin may also suffer strand scission at the sites of reacting thymine upon heating under the alkaline conditions as represented by Scheme 1.

Scheme 1. Schematic Representation of the Proposed Mechanism for Photocrosslinking in Nucleosome



It has been reported that UV irradiation of chromatin produces DNA-histone adducts and that the degree of formation of each adducts may be influenced by wavelength of light.^{3,7} Several investigations have also demonstrated which histones are preferentially cross-linked to DNA. Recently, cross-linked DNA-histone adducts formed in 254-nm irradiation of chicken erythrocyte nuclei were isolated and purified.^{3 3} On the other hand, Kunkel and Martinson⁸ have observed that a considerable amount of reversal of DNA-histone cross-links occurred in irradiation of calf thymus nuclei with light between 230 and 290 nm, *i.e.*, the histone cross-linked to DNA are released by chemical hydrolysis or upon rechromatography on Sepharose column, whilst the chemical nature of the reversibility and the sites of binding have been totally unknown. We also examined the DNA-histone crosslinks induced by the known method. External irradiation of the nuclei (0.1 mg/mL) in buffer A (NaHCO_3 - Na_2CO_3 -EDTA, pH 9.5) was performed at 0°C under a nitrogen atmosphere with a 400 W high-pressure mercury lamp through Vycor filter (>250 nm). Separation of crosslinked histone-DNA adducts was conducted by the standard procedure outlined in Chart 1. After adding SDS to the irradiated solutions to 2% (V/V), the sample were ultracentrifuged to remove free histones. High molecular weight materials (mainly histone-DNA adducts) were precipitated and resuspended in buffer A. Further ultracentrifuged gave histone-DNA adducts as pellets which were dissolved in buffer A and heated at 90°C for 2 h to ensure the conversion of 3 to 2 (Eq. 1). Histones were analyzed by polyacrylamide gel electrophoresis (PAGE) on 2 mm thick SDS slab gels. As shown in Fig. 4, free histones present in supernatants were decreased with increasing irradiation time (Fig. 4, a). Upon being heated at 90°C for 2 h, the histone-DNA adducts released free histones which were detected by SDS PAGE (Fig. 4, b). By knowing the chemical reactivity of 3 and by the confirmation of 2 in UV-irradiated DNA-histone complexes, it is now evident that the apparent reversibility is mainly due to the cross-links of the lysine ϵ -amino groups to thymine in DNA, although the details should await further studies using

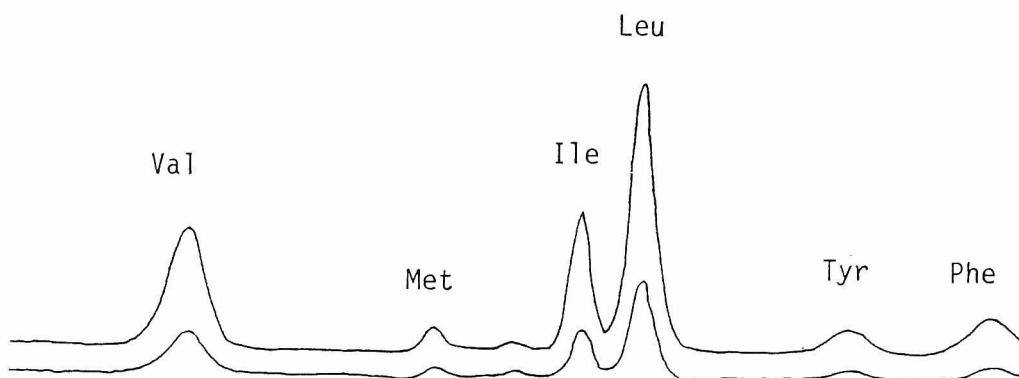


Figure 3. Output from amino acid analyzer for the hydrolysate of irradiated chicken erythrocyte nuclei.

Chart 1.

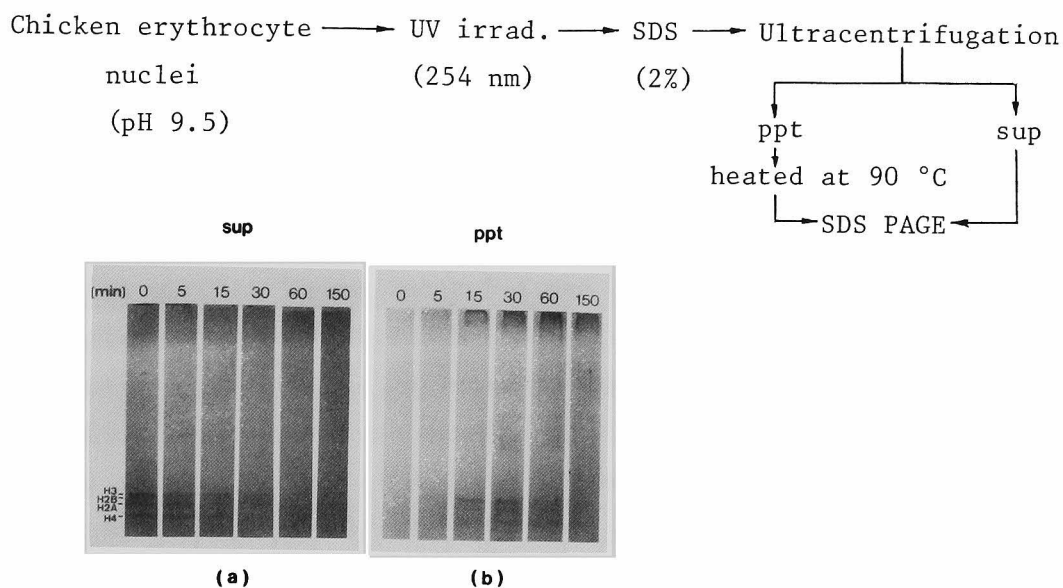


Figure 4. Histone patterns on SDS/polyacrylamide gel electrophoresis. Equivalent volumes of nuclei were withdrawn from the irradiated sample, pelleted, dissolved in buffer A and heated at 90 °C for 2 h. (a) Supernatant; (b) Precipitate. The gel was stained with Coomassie Brilliant Blue.

mine in DNA, although the details should await further studies using each of the purified histone-DNA adducts.

In conclusion, the present work has demonstrated an important role of thymine-lysine photoreaction in the photo-cross-linking of histones to DNA in chromatin. Since the thymine-lysine photoadduct 2 is readily detectable by HPLC and amino analyzer, irradiation with 254-nm light at a slightly alkaline pH provides a useful means for probing specific interaction sites between thymine and lysine residues in DNA-protein complexes. Furthermore, such a type of photocross-linking may be relevant to the UV-induced damage on nucleic acid in cells.

EXPERIMENTAL

Ultraviolet spectra were recorded with a Shimadzu UV-200 spectrophotometer. Protonmagnetic resonance spectra were recorded with a JEOL JNM-GX400 spectrometer using Me_4Si as the internal reference. High-performance LC (HPLC) was performed on a Waters ALC/GPC 204 model equipped with a 254 nm fixed wavelength detector. Reverse-phase μ -Bondapak C_{18} (1 x 25 cm) column were used for analytical and preparative HPLC, respectively. Amino acid analysis was performed on a Hitachi Amino Acid Analyzer, Model KLA 3B. Ultracentrifuge was performed on a Beckman L-50B model. Irradiations were carried out by using a 400 W high-pressure mercury lamp. Vycor filters were used to cutoff wavelengths of $\lambda < 240$ nm in all cases. Calf thymus nucleohistone was purchased from Sigma as lyophilized powder (DNA 40%, proteins 54%) and used without purification. Its spectral ratios were as follows: $A_{260}/A_{230} = 1.53$; $A_{260}/A_{280} = 1.83$; max/min = 1.69; $A_{260}/A_{320} = 116$. The proteins contains lysine (22.6%) and arginine (10.7%) as determined by amino acid analyzer. Chicken erythrocyte nuclei were prepared from chicken erythrocyte according to Rill et al.¹⁰ We are indebted to Professor M. Yanagida, Kyoto university, for providing us the sample.

Irradiation of Calf Thymus Nucleohistone at Various pH.

A solution of calf thymus nucleohistone (50 mg) in 5 mM NaHCO_3 - Na_2CO_3 buffer (200 mL) was adjusted to appropriate pH and irradiated with a 400 W medium-pressure mercury lamp through Vycor filter (>240 nm) at 10 °C under nitrogen atmosphere. After irradiation the solution was heated at 70 °C for 2 h and then lyophilized to dryness. The residue was dissolved in 6 N HCl (5 mL), and the solution was heated at 110 °C in a sealed tube for 22 h. The hydrolysate was evaporated to dryness in vacuo, and the residue was co-evaporated with water (3 x 5 mL), then dissolved in water (5 mL) and subjected to HPLC analysis (0.5% aqueous acetic acid, 4 mL/min) and amino acid analysis. The amount of 2 with retention time of 21.2 min were determined by HPLC. Identification of 2 was made by comparison of

the chromatographic behaviors (TLC, HPLC) and UV and ^1H NMR spectra with those of authentic sample after collection of the HPLC fraction.

Irradiation of Chicken Erythrocyte Nuclei.

A solution of chicken erythrocyte nuclei (0.5 mg) in 5 mL of buffer A (5 mM NaHCO_3 , 5 mM Na_2CO_3 , 0.5 mM EDTA, pH 9.5) was irradiated with a 400 W medium-pressure mercury lamp through Vycor filter (>240 nm) at 0°C externally. After irradiation the solution was heated at 70°C for 2 h and then lyophilized to dryness. The residue was dissolved in 6 N HCl (2 mL), and the solution was heated at 110°C in a sealed tube for 22 h. The hydrolysate was evaporated to dryness in vacuo, and the residue was co-evaporated with water (3 x 2 mL), then dissolved in water (1 mL) and subjected to amino acid analysis.

Detection of Free Histone Release from Crosslinked Histone-DNA Adducts.

A solution of chicken erythrocyte nuclei (0.5 mg) in buffer A (5 mL) in quartz test tube was adjusted to pH 9.5 and irradiated externally with a 400 W medium-pressure mercury lamp through Vycor filter (>240 nm) at 10°C for various irradiation time listed in Figure 3. After irradiation a 20% SDS solution was added to irradiated samples to 2% (V/V), and the samples were ultracentrifuged (200000 g) for 1 h. Resulting pellets were resuspended in buffer A (5 mL) and were ultracentrifuged as described above. Resulting pellets were dissolved in buffer A (0.5 mL) and heated at 90°C for 2 h, and then subjected SDS gel electrophoresis. Electrophoresis was carried out in SDS discontinuous 15% polyacrylamide slab gels by using the buffer system of Laemmli.¹¹ The gel were stained with 0.1% Commassie Brilliant Blue in 50% methanol-10% acetic acid and destained in 10% acetic acid.

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CHAPTER 6

Stacking Interactions between Pyrimidine Bases and Indole Rings in Synthetic Model Compounds for Nucleic Acids-Protein Interactions

ABSTRACT

As a model for the stacking interaction between nucleic acid bases and tryptophan in a protein, several compounds (3, 4, 7, 9 and 11) possessing a pyrimidine base and an indole ring in the same molecule were synthesized. Their absorption and fluorescence spectra and X-ray crystallographic analysis of 11 were examined. The spectral results indicated that the stacking interaction between the two rings is strong in 7 and 9, moderate in 3 and 11, and weak in 4. In a crystalline state there is an intermolecular stacking interaction between the indole ring and the uracil ring of the model compound 11.

INTRODUCTION

It has been recognized that the specific interactions for the recognition of proteins to nucleic acids involve electrostatic forces, hydrogen bonding and stacking interactions,¹ all of which depend upon the accomodating sizes, shapes and spacing of the interacting units. Among such specific stacking interactions,² that of tryptophan or related indolic compounds with nucleic acid bases have been demonstrated, (1) by reflectance and luminescence studies of aggregates formed between tryptophan and nucleic acid components in aqueous frozen solutions,³ (2) by the hypochromism and the quenching of indole fluorescence in synthetic spectroscopic models⁴ and (3) by the X-ray structural and conformational studies of model compounds⁵.

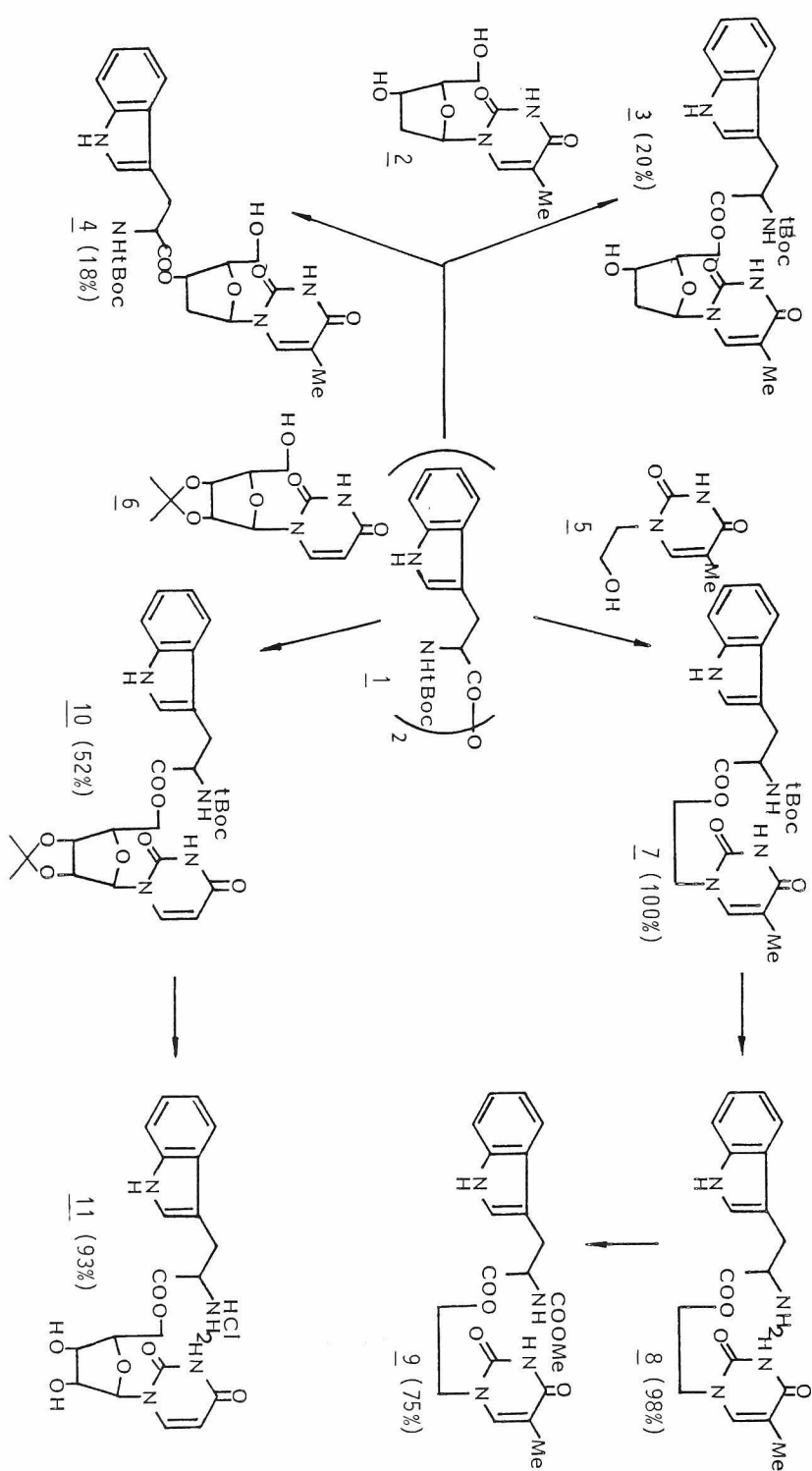
In order to know the nature of the stacking interaction between pyrimidine base and tryptophan, model compounds having an indole and a pyrimidine ring in the same molecule were synthesized, and their interactions were investigated by absorption and fluorescence spectroscopy as well as X-ray crystallographic analysis.

RESULTS AND DISCUSSION

Synthesis of Model Compounds.

Synthetic scheme for the preparation of model compounds (3, 4, 7, 9 and 11) is illustrated in Scheme 1. Compounds 3 and 4 were synthesized by the treatment of t-Boc-tryptophan anhydride 1⁶ with thymidine. 3 and 4 were separated by preparative TLC and the assignment of their structures were made on the bases of their ¹³C-NMR spectra. Treatment of 1 with 1-(2-hydroxyethyl)thymine 5 gave 7 which was converted to 9 by selective hydrolysis followed by methoxycarbonylation. Compound 11 was synthesized by the treatment of 1 with 2',3'-O-isopropylideneuridine 6 followed by selective hydrolysis. The yields for each steps are listed in Scheme 1.

Scheme 1



Absorption Spectroscopy

One criterion of assessing the interaction between the two rings is the hypochromic effect which depends on the degree and orientation of intramolecular stacking. In order to examine the interaction between the indole and pyrimidine bases of model compounds (3, 4, 7, 9 and 11), their UV absorption spectra were quantitatively compared with those of the 1 : 1 mixtures of their parents components in each systems (Table 1). This method had been used for assessing the stacking interactions between nucleic acid bases and coenzyme moieties.^{6,7} Figure 1 shows the UV absorption spectra of 11 and of the 1 : 1 mixtures of tryptophan methyl ester and uridine in acetonitrile. The spectrum of 11 exhibits a remarkable decrease in the absorption at 260-290 nm region. This decrease in absorption is caused by the hypochromic effect, indicating a stacking interaction between indole and uracil rings in a solution of 11. The percent hypochromism, H (%), of (3, 4, 7, 9 and 11) in acetonitrile at room temperature was calculated according to the following equations^{7a} and the results are given in Table 1.

Table 1 Calculated Percent Hypochromism for the 240-300 nm Absorption Band^a

	<u>3</u>	<u>4</u>	<u>7</u>	<u>9</u>	<u>11</u>
H (%)	6.0	3.0	22.9	28.1	11.1

^a The parent components are: *t*-Boc-tryptophan methyl ester and 3',5'-diacetylthymidine for 3 and 2; *t*-Boc-tryptophan methyl ester and 1-(2-acetoxyethyl)thymine for 7; N^β-methoxycarbonyltryptophan methyl ester and 1-(2-acetoxyethyl)thymine for 9; and tryptophan methyl ester and uridine for 11.

$$H (\%) = \left\{ 1 - \frac{f_{AB}}{f_A + f_B} \right\} \times 100$$

$$f = 4.32 \times 10^{-9} \int \{ \epsilon(\lambda) / \lambda^2 \} d\lambda$$

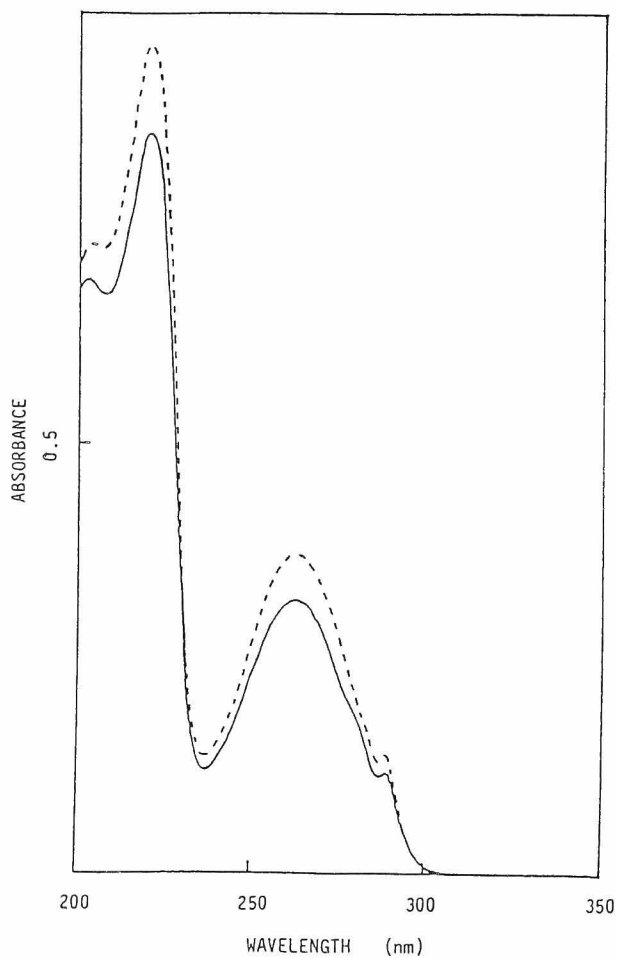


Figure 1. UV spectra of acetonitrile solutions of 11 (—) ($3 \times 10^{-5}M$) and of equimolar mixture (---) of tryptophan methyl ester (each $3 \times 10^{-5}M$) and uridine.

All of the model compounds do show appreciable hypochromism which is indicative of the presence of intramolecular interaction between the indole and the pyrimidine base due to the stacked conformations. Although the magnitude of H (%) is depending upon the thermodynamic strength of the interaction⁸ and the relative orientation of the transition moments,⁹ it may be used as an appropriate measure of the strength of interaction. The H (%) values indicate that the compounds linked by ribose such as 3, 4 and 11 may interact weakly in an intramolecular fashion compared to the compound linked by the alkyl ester chain like 7 and 9. The smallest value for 4 may be due to the extended character of 3'-substituent of the thymidine part. The higher H (%) for 9 than that for 7 suggests that 9 is stacked to higher extent than 7, probably due to its smaller protecting group of the α -amino group.

Fluorescence Spectroscopy

The intramolecular fluorescence quenching of the indole moiety by the pyrimidine base in the model compound 3, 4, 7, 9 and 11 was studied in acetonitrile at room temperature. Figure 2 shows the fluorescence spectra of 11 ($3 \times 10^{-5} M$, acetonitrile) and of the equimolar mixture of its parent components. The relative quenching efficiencies, q^{rel} , given in Table 2, were determined by comparing the fluorescence intensity of each model compound with that of a 1 : 1 mixture of the parent components. Both spectra had an equal absorbance at the excitation wavelength (290 nm). In all cases appreciable quenching of indole fluorescence by the attached pyrimidine base was observed. This quenching may result from either of the following two processes: (1) static quenching, time-independent process resulting from the proximity of the quencher predating excitation, or (2) dynamic quenching, a time-dependent process resulting from the quenching during the excited state lifetime. The quenching process of 11 is illustrated in Scheme 2. In acetonitrile 11 may exist in a population of conformations including extended and stacked

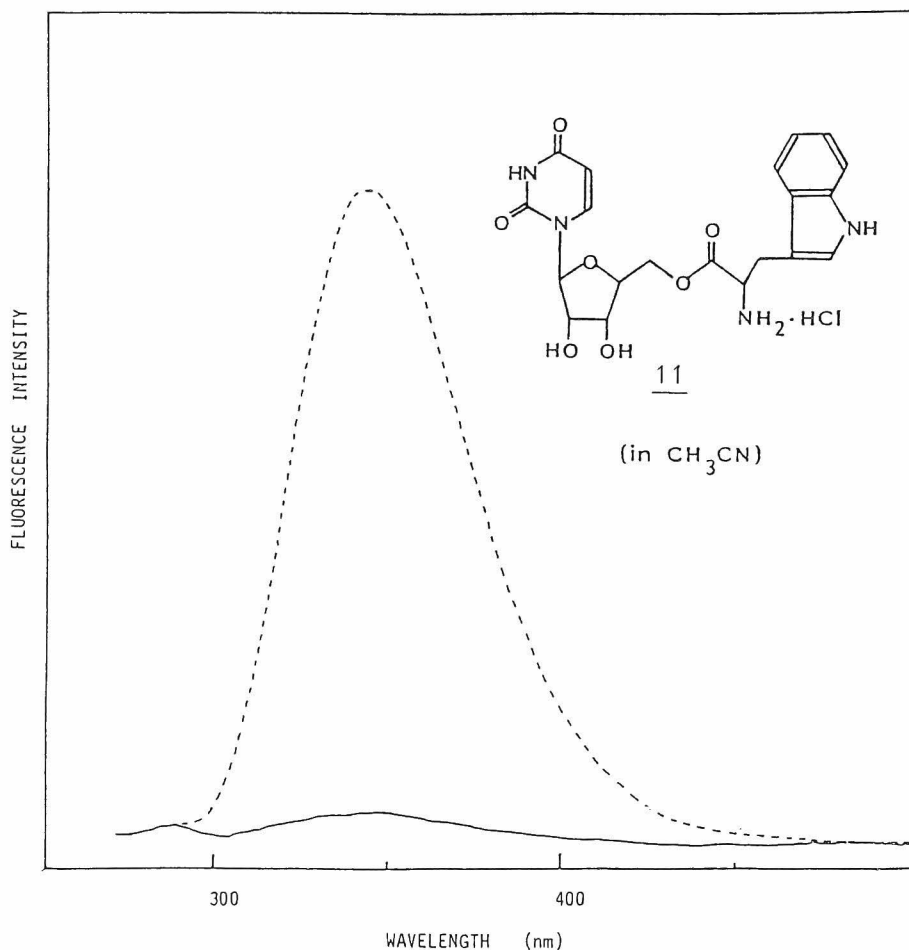


Figure 2. Fluorescence spectra of acetonitrile solutions of 11 (—, 3×10^{-5} M) and equimolar mixture (each 3×10^{-5} M) of Tryptophan methyl ester and uridine (---) at room temperature.

forms. Irradiation of those molecules that are intramolecularly stacked results in immediate quenching with no fluorescence. Direct fluorescence emission of indole ring is observed only from the extended form. For this reason the q^{rel} can be used as a measure for the strength of stacking interaction. The results of fluorescence quenching are roughly in accordance with those of UV hypochromism, strongest quenching being observed in the case of 9.

Scheme 2

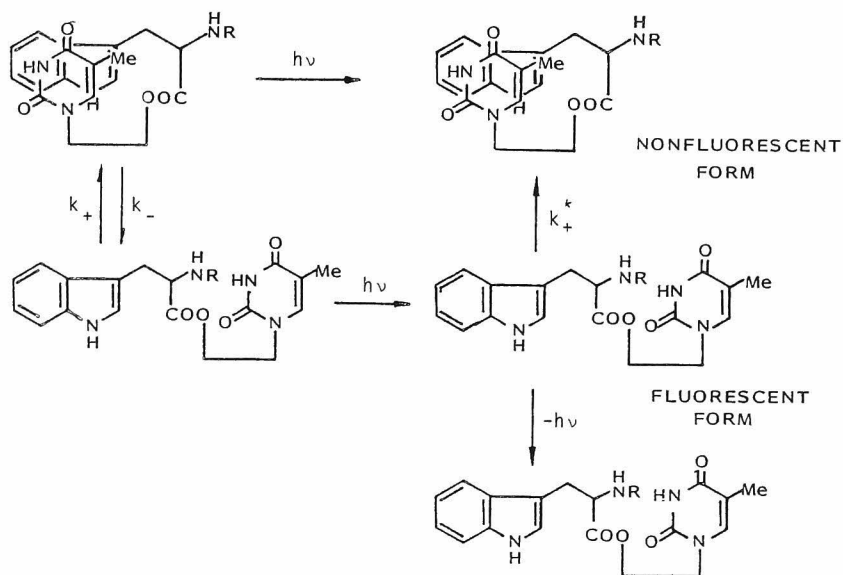


Table 1. Fluorescence Quenching by Intramolecular Stacking of the Model Compounds (3, 4, 7, 9 and 11).

	<u>3</u>	<u>4</u>	<u>7</u>	<u>9</u>	<u>11</u>
q^{rel}	0.075	0.340	0.072	0.034	0.041

X-ray crystallographic analysis

Specific interaction between indole ring and pyrimidine base was observed in spectroscopic studies, but direct structural information is not available from these studies. In order to elucidate a fundamental feature of the interaction between uracil ring and tryptophan, the crystalline structure of 11 was determined by an X-ray analysis. Crystallographic data for 11 are shown in Table 3.

Table 3. Crystallographic Data for 11

molecular formula	$C_{20}H_{22}N_4O_7 \cdot HCl \cdot H_2O$
unit cell dimension	$a = 11.694 (3) \text{ \AA}$ $b = 6.948 (2) \text{ \AA}$ $c = 13.325 (3) \text{ \AA}$
space group	$P 2_1$
Z	2
ρ_{calcd}	1.50 g cm^{-3}

Refinement resulted in a conventional R value of 0.04. The molecular structure of 11 as well as the atomic numbering system used in this chapter are illustrated in Figure 3, and the coordination of non-hydrogen atoms of the structure are given in Table 2.

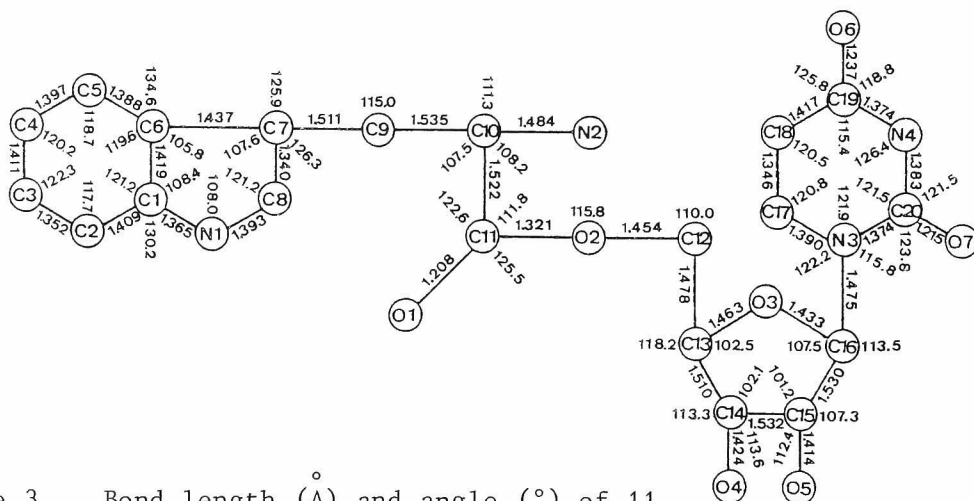


Figure 3. Bond length ($\text{\AA}$) and angle ($^\circ$) of 11.

The dimensions of the uracil ring closely resemble with those found in other uracil-containing structures.¹⁰ The bond length and angles of the indole group are within their expected ranges. Figure 4 shows the stereoscopic view of the conformation of 11 in crystals.

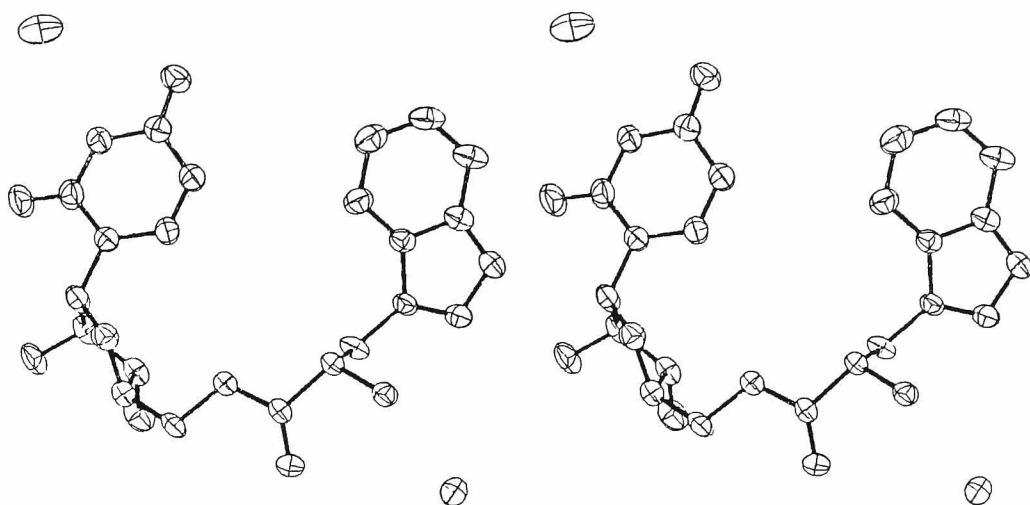


Figure 4. Stereoscopic view of the conformation of 11.

The ribose unit has C3' endo conformation and orientation of the base with respect to the sugar is *anti*. The overlapping mode between the two molecules related by a center of symmetry is shown in Figure 5 in which 11 exhibits a extended conformation, and the indole ring is stacked on the uracil group of yet another neighbouring molecule. The stacking between the uracil and indole rings is illustrated in Figure 6.

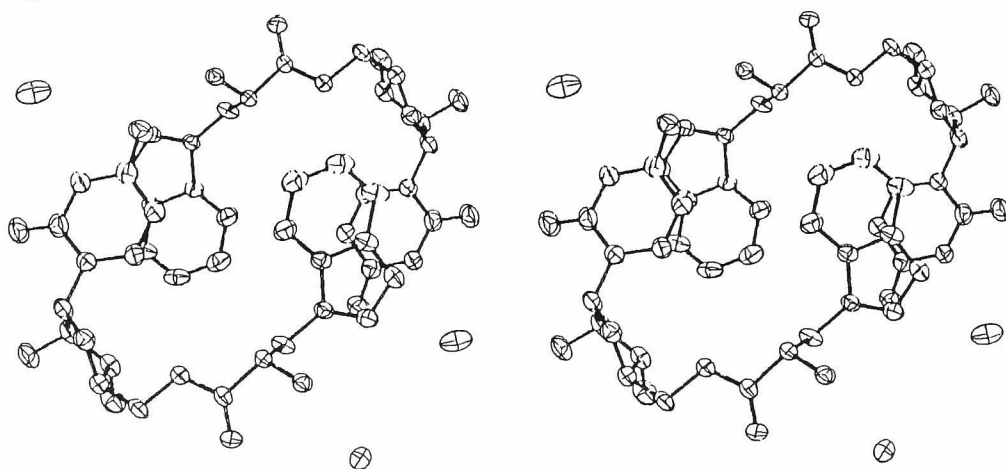


Figure 5. The overlapping mode between two molecules related by a center of symmetry.

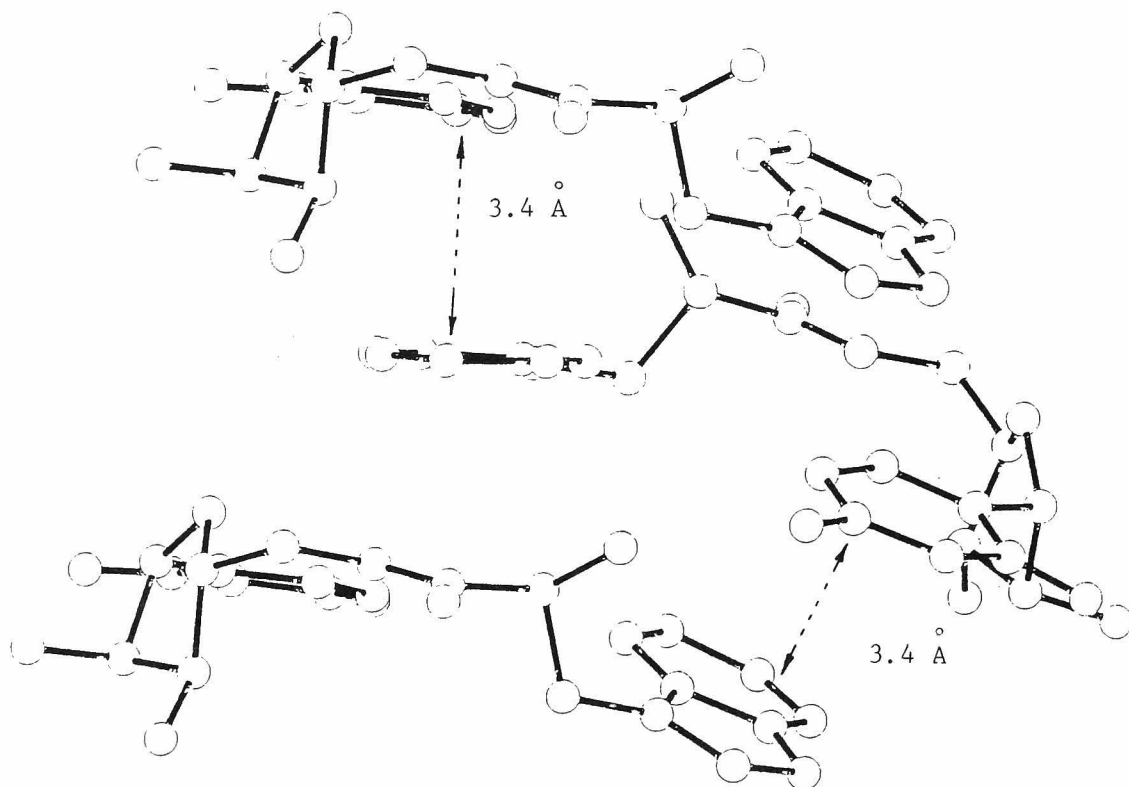


Figure 6. Stacking between the indole ring of 11 and its two nearest neighboring uracil rings projected parallel to the ring.

Both the upper and lower indole rings are well stacked on the central uracil ring as seen in Figure 6. The average interplanar spacing in the overlap region is about 3.4 Å. This value is in the normal van der Waals separation.

From the spectroscopic study of model compounds in solution, the folded conformation with a stacking interaction between the indole and pyrimidine rings was suggested to be strong. In the crystalline state, conformation of 11 was an extended form, although the indole moiety was associated with the uracil ring of the adjacent molecule through a normal stacking interaction. This might be caused by the packing interaction between the neighboring molecules. These results indicate that the indole ring of the tryptophan residue can selectively interact with uracil or thymine in nucleic acid-protein systems.

Therefore, such a stacking force between the indole ring of a tryptophan residue and a nucleic acid base may play an important role in the specific recognition between proteins and nucleic acids.

EXPERIMENTAL

Melting points are uncorrected. Elemental analysis were performed at the Analytical Center of Kyoto University. Ultraviolet spectra were recorded on a Shimadzu UV-200 spectrophotometer. For quantitative measurement 3.3×10^{-5} M solution of substrates in acetonitril were prepared. Fluorescence spectra were recorded on a Shimadzu RF-500 spectrophotometer. Proton magnetic resonance spectra were recorded with a Varian HA-100 or a Varian T-60 spectrometer using Me_4Si as the internal reference. Carbon-13 magnetic resonance spectra were recorded with a Varian FT-80A spectrometer. Column chromatography and preparative TLC were carried out on Wako silica gel C-200 and Merck 60 PF₂₅₄, respectively.

N^α -*t*-Butoxycarbonyl-L-tryptophan anhydride (1). The compound 1 was prepared by the procedure of Henderson *et al.*⁶ using N^α -*t*-butoxycarbonyl-L-tryptophan (3.04 g, 10 mmol) and dicyclohexylcarbodiimide (1.14 g, 5.5 mmol) in ethyl acetate. The product was obtained as white powder (2.77 g, 88%). 1; mp. 122 - 124 °C; $^1\text{H-NMR}$ (CDCl_3) δ 1.40 (s, 18 H), 3.18 (brd, 4 H), 4.6 (m, 1 H), 6.7 - 8.2 (m, 10 H).

5'-Thymidylyl- N^α -*t*-butoxycarbonyl-L-tryptophanate (3) and 3'-thymidylyl- N^α -*t*-butoxycarbonyl-L-tryptophanate (4). The compounds (3) and (4) were prepared by the application of the method of Henderson *et al.*⁶ A solution of 1 (300 mg, 0.5 mmol) in anhydrous pyrimidine (2 mL) was added to a cooled (0°C), stirred solution of thymidine 2 (121 mg, 0.5 mmol) in pyridine (4 mL). The solution was stirred for 4 h while being allowed to come to room temperature. After addition of water (5 mL), the solution was stirred for 20 min and the solvent was removed in vacuo. The residue was partitioned between ethyl acetate (30 mL) and water (30 mL), and the organic layer was dried over sodium sulfate and concentrated in vacuo. The residue was chromatographed on a silica gel column with ethyl acetate-hexane (1 : 1). The two major fractions (R_f 0.5 and 0.4) were separated to produce 3 (53 mg, 20%) and 4 (48 mg, 18%), respectively. 3: colorless fine crystals;

mp. 123–125 °C (dec, reprecipitated from CHCl₃-pentane); UV (CH₃CN) 267 nm (log ε 4.11), emission maximum (CH₃CN) 340 nm (Excited at 290 nm); ¹H NMR (CDCl₃) δ 1.42 (s, 9H), 1.91 (br s, 3H), 2.8–3.6 (m, 3H), 3.93 (br s, 1H), 4.60 (m, 1H), 5.36 (m, 1 H), 6.14 (m, 1H), 6.9–7.7 (m, 6 H), 8.98 (br s, 1 H), 9.88 (br s, 1 H); ¹³C NMR (CDCl₃) δ 12.9 (q), 28.9 (q), 29.3 (t), 40.6 (t, C-2'), 56.5 (d), 65.9 (t, C-5'), 72.4 (d, C-3'), 80.9 (s), 85.5 (d, C-1'), 86.3 (d, C-4'), 110.5 (s), 111.9 (s), 112.7 (d), 119.1 (d), 120.1 (d), 122.8 (d), 124.5 (d), 128.4 (s), 137.0 (s), 138.0 (s), 152.0 (s), 157.2 (s), 166.2 (s), 174.4 (s) ppm. 4: colorless fine crystals. mp. 125–125 °C (dec, reprecipitated from CHCl₃-pentane), UV (CH₃CN) 267 nm (log ε 4.13), emission maximum (CH₃CN) 340 nm (Excited at 290 nm); ¹H NMR (CDCl₃) δ 1.44 (s, 9 H), 1.86 (br s, 3 H), 1.8–2.3 (m, 2 H), 3.26 (m, 2 H), 3.66 (br s, 2 H), 4.62 (m, 1 H), 5.1–5.4 (m, 2 H), 5.74 (m, 1 H), 7.0–7.7 (m, 6 H), 9.0 (br s, 1 H); ¹³C NMR (CDCl₃) δ 12.4 (q), 26.9 (t), 28.0 (q), 36.9 (t, C-2'), 55.3 (d), 61.5 (t, C-5'), 75.7 (d, C-3'), 78.8 (s), 84.0 (d, C-1'), 84.8 (d, C-4'), 109.8 (s), 110.0 (s), 111.7 (d), 118.3 (d), 118.7 (d), 121.3 (d), 124.0 (d), 127.4 (s), 136.0 (d), 136.3 (s), 150.7 (s), 155.7 (s), 163.9 (s), 172.2 (s) ppm.

2-(1'-Thyminy)ethyl-N^α-*t*-butoxycarbonyl-L-tryptophaniate (7).

The compound 7 was prepared from 1-(2-hydroxyethyl)thymine 5 and 1 by the same procedure as described for the preparation of 3 and 4 in almost quantitative yield. 7: colorless crystal. mp. 198–200 °C; UV (CH₃CN) 268 nm (log ε 4.02), emission maximum (CH₃CN) 340 nm (Excited at 290 nm); ¹H NMR (CDCl₃) δ 1.30 (s, 9 H), 1.72 (s, 3 H), 3.00 (m, 2 H), 3.80 (m, 3 H), 4.22 (m, 3 H), 6.80–7.60 (m, 6 H), 10.80 (m, 1 H), 11.30 (m, 1 H); Mass spectrum (rel. intensity) m/e 456 (M⁺, 4), 400 (5), 556 (6), 339 (8), 269 (19), 268 (96), 200 (40), 171 (27), 130 (100), 129 (70), 53 (100). Exact mass. Found: 456.2025. Calcd. for C₂₃H₂₈N₄O₆: 456.2008.

2-(1'-Thyminy)ethyl-N^α-methoxycarbonyl-L-tryptophaniate (9).

A solution of 7 (460 mg, 1 mmol) in 100% formic acid (50 mL) was stirred at room temperature for 3 h. The solution was evaporated

in vacuo to give a viscous yellow oil. This was dissolved in methanol (2 mL) and added dropwise to cold ethyl acetate (20 mL) under stirring. The precipitated white powder was collected and dried in vacuo to give 376 mg of 8 (92%). 8: white powder; ^1H NMR ($\text{DMSO}-d_6$) δ 1.77 (s, 3 H), 2.0–2.4 (m, 2 H), 3.1–4.4 (m, 7 H), 5.29 (br s, 1 H), 6.12 (t, 1 H, $J = 8$ Hz), 6.9–7.8 (m, 6 H), 8.74 (br, 3 H), 11.10 (br s, 1 H), 11.27 (s, 1 H); Anal. Calcd. for $\text{C}_{21}\text{H}_{24}\text{O}_6\text{N}_4 \cdot \text{HCl} \cdot 2\text{H}_2\text{O}$: C; 50.35, H; 5.84, N; 11.18, Cl; 7.08. Found: C; 50.50, H; 5.85, N; 11.16, Cl; 7.12.

A solution of 8 (89 mg, 0.25 mmol) in dioxane (10 mL) and ethyl acetate (10 mL) was cooled to 0 °C. Trimethylamine (25.3 mg, 25 mmol) and methyl chloroformate (27 mg, 0.28 mmol) in 5 mL of dioxane were added, and the solvent was removed in vacuo. The residue was partitioned between ethyl acetate (50 mL) and water (50 mL), and the organic layer was separated, dried and concentrated in vacuo. The residue was subjected to a silicagel preparative TLC with ethyl acetate to give 9 (31 mg, 75%). 9: colorless fine crystals; mp. 164 – 165.5 °C; UV (CH_3CN) 264 nm ($\log \epsilon$ 4.00); emission maximum (CH_3CN) 340 nm (Excited at 290 nm); ^1H NMR (CDCl_3) δ 1.78 (s, 3 H), 3.32 (m, 2 H), 3.62 (s, 3 H), 4.20 (m, 2 H), 6.80–7.80 (m, 6 H), 8.20 (m, 1 H), 8.95 (m, 1 H); Mass spectrum (rel. intensity) m/e 414 (M^+ , 35), 399 (32), 381 (13), 187 (24), 153 (59), 154 (58), 131 (100), 130 (100), 82 (16), 81 (17). Exact mass. Found: 414.1518. Calcd. for $\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_6$: 414.1539.

5'-(2',3'-O-Isopropylidene)uridinyl- N^α -*t*-butoxycarbonyl-L-tryptophanate (10). The compound 10 was prepared from 2',3'-O-isopropylideneuridine and 1 by the same procedure as described for the preparation of 1 and 2 in 52% yield. 10: colorless fine crystals; mp. 125–127 °C; ^1H NMR (CDCl_3) δ 1.31 (s, 3 H), 1.41 (s, 9 H), 1.52 (s, 3 H), 3.24 (m, 2 H), 4.40–4.80 (m, 6 H), 5.22 (m, 1 H), 5.6–5.7 (m, 2 H), 6.9–7.7 (m, 6 H), 8.42 (br s, 1 H), 9.22 (br s, 1 H); ^{13}C NMR (CDCl_3) δ 25.3, 27.0, 27.1, 28.1, 54.5, 64.3, 78.7, 80.1, 84.2, 93.3, 102.4, 109.8, 111.3, 114.3, 118.3, 119.4, 122.0, 123.0, 127.3, 136.2, 141.3,

150.0, 155.1, 163.2, 172.3 ppm; Anal. Calcd. for $C_{28}H_{34}O_9N_4$: C; 58.94, H; 6.01, N; 9.84. Found: C; 58.50, H; 6.02, N; 9.65.

5'-Uridyl-L-tryptophanate hydrochloride (11). A solution of 10 (570 mg, 1 mmol) in methanol (100 mL) and conc. HCl (3 mL) was stirred at room temperature for 6 h. The solution was evaporated in vacuo to give a viscous yellow oil. The colorless crystal obtained was recrystallized from methanol to give 11 as the monohydrochloride salt (450 mg, 93%). 11: colorless fine crystals, mp. 174-176 °C; UV (MeOH) 291 nm ($\log \epsilon$ 3.73) 265 nm (4.13); emission maximum (CH_3CN) 351 nm (Excited at 290 nm); 1H NMR ($DMSO-d_6$) δ 3.20-3.50 (m, 2 H), 3.80-4.50 (m, 6 H), 5.20-5.60 (m, 2 H), 5.64 (d, 1 H, $J = 8$ Hz), 5.76 (d, 1 H, $J = 4$ Hz), 6.90-7.70 (m, 6 H), 7.58 (d, 1 H, $J = 8$ Hz), 8.50-9.00 (m, 3 H), 11.10 (br, 1 H), 11.34 (br, 1 H); ^{13}C NMR ($DMSO-d_6$) δ 26.4 (t), 53.1 (d), 65.4 (t), 69.6 (d), 72.7 (d), 80.9 (d), 89.3 (d), 102.4 (d), 106.6 (s), 111.9 (d), 118.3 (d), 118.9 (d), 121.4 (d), 125.2 (d), 127.0 (s), 136.4 (s), 141.3 (d), 150.8 (s), 163.3 (s), 169.3 (s) ppm; Anal. Calcd. for $C_{20}H_{22}O_4N_7 \cdot HCl \cdot H_2O$: C; 49.54, H; 5.10, N; 11.56, Cl; 7.32. Found: C; 49.72, H; 5.32, N; 11.49, Cl; 7.29.

X-ray Analysis. The compound 5 was crystallized from methanol as colorless plate. Diffraction data were measured on a microcomputer-controlled for four-circle diffractometer.¹¹ The unit cell dimensions were derived from a least-squares fit to the observed values of 2θ for 21 reflections. The crystal data are as follows: $C_{20}H_{22}N_4O_7 \cdot HCl \cdot H_2O$, $M = 484.9$, monoclinic, space group $P2_1$, $a = 11.694(3)$, $b = 6.948(2)$, $c = 13.325(3)$ Å, $\beta = 97.55(2)^\circ$, $Z = 2$, $V = 1073.2$ Å³, $D_c = 1.50$ g.cm⁻³, $\mu(CuK\alpha) = 20.9$ cm⁻¹. Of 1723 independent reflections measured up to $2\theta = 120^\circ$ by a $\theta - 2\theta$ scan technique using Ni-filtered $CuK\alpha$ radiation, 1645 reflections whose intensities were than $2\sigma(I)$ were used for subsequent calculations. A periodically monitored reflections showed no significant change in intensity. The intensities were corrected only for the Lorentz and polarization factors.

The structure was solved by the direct method¹² using 250 reflec-

tions. The E-map based on the phase set of the highest combined figure of merit gave a fragment of the molecule. The remaining non-hydrogen atoms were located in the successive Fourier synthesis. Positional and anisotropic temperature factors were refined by the block-diagonal least-squares method.¹³ Twenty-two out of twenty-five hydrogen atoms were located in the difference Fourier synthesis and included in the refinement. The weighting scheme was $w = 1.0$ for $F \leq 10$ and $w = [1.0 + 0.13(F_o - 10)]^{-1}$ for $F_o > 10$. The final R value was $w = 1.0$ for 1643 reflections. The atomic scattering factors were taken from ref. 14. The calculations were performed on a HITAC M-150 computer at the Tottori University Computing Center.

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CHAPTER 7

Photochemical Reaction between Tryptophan and Pyrimidine Bases

ABSTRACT

Irradiation of tryptophan and thymine in aqueous solution gave a novel type of adducts 3 and 4. The structures of 3 and 4 were determined by spectral data and X-ray crystallographic analysis. Irradiation of tryptophan and uracil gave 6 and 7 in a similar fashion. Under the irradiation conditions indole-3-acetaldehyde reacted with thymine to provide 3 and 4. Photoaddition of acetone to tryptophan is also described.

INTRODUCTION

UV irradiation is known to cause serious effects to biological systems. Cell killing or mutation for bacteria and skin cancer induction for human are typical examples. Many reports have indicated that stable covalent cross-linking between DNA and protein are formed, when cells are irradiated with UV light.¹ In order to understand the nature of photo-cross-linking of DNA to protein, we are exploring the structures and mechanisms of formation of adducts of nucleic acid bases with amino acids.² We have already found that photoexcited thymidine reacts with lysine to produce a ring-opened adduct, which on subsequent heating is readily converted to an N(1)-substituted thymine.³ We also confirmed that this type of photo-reaction between lysine in histone and thymine in DNA is occurring in chicken erythrocyte nuclei.⁴

In most of these studies concerning the model system of photo-crosslinking, the absorption of light by the nucleic acid base appears to be the primary event, and very little is known about the photoreaction between nucleic acid bases and aromatic amino acids. The only study in which products have been fully characterized is that of photocoupling reaction of bromouracil derivatives to tryptophan derivatives.⁵ In a previous chapter we revealed that there is a considerable stacking interaction between the pyrimidine base and indole ring. Photochemistry of tryptophan and pyrimidine bases is an attractive one in this point of view.

Recently, Reeve and Hopkins⁶ have reported that irradiation of thymine and tryptophan in an aqueous buffered solution at pH 7.0 with filtered light (>260 nm) gave two thymine-tryptophan adducts of 1 : 1 molar ratio. We independently found that irradiation of an aqueous solution of a mixture of thymine and tryptophan gave a 1 : 1 adducts whose structures were determined by 400 MHz ¹H NMR spectroscopy as well as X-ray crystallographic analysis. In this chapter, we describe the photoreaction of tryptophan with thymine and uracil, and the reaction mechanism of this photoreaction is also discussed.

Results and Discussion

An aqueous solution (200 mL) of tryptophan (1, 204 mg, 1 mmol) and thymine (2, 25 mg, 0.2 mmol) was irradiated with a 10 W low -pressure mercury lamp through Vycor filter (>250 nm) at room temperature for 12 h. High performance LC (HPLC) analysis of the photolysate revealed the formation of two photoproducts 3 and 4. The profile of the photolysate on HPLC was shown in Figure 1.

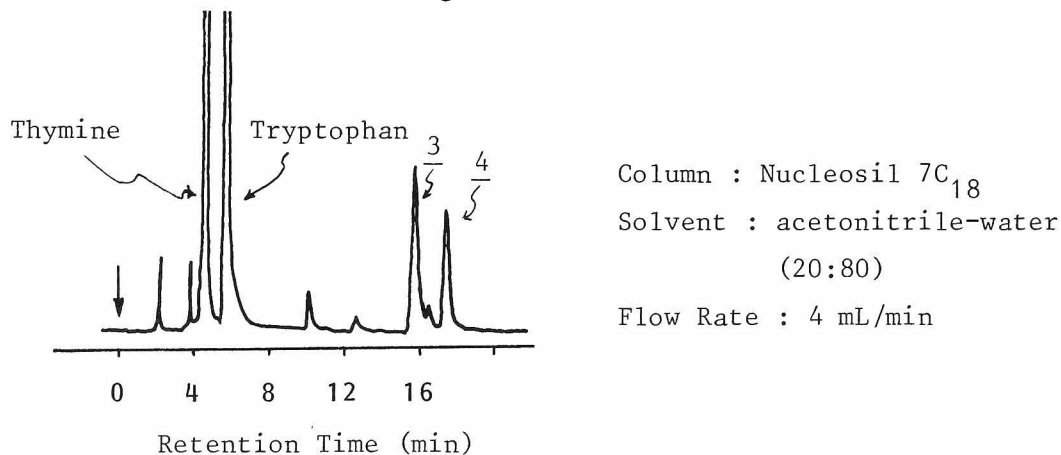
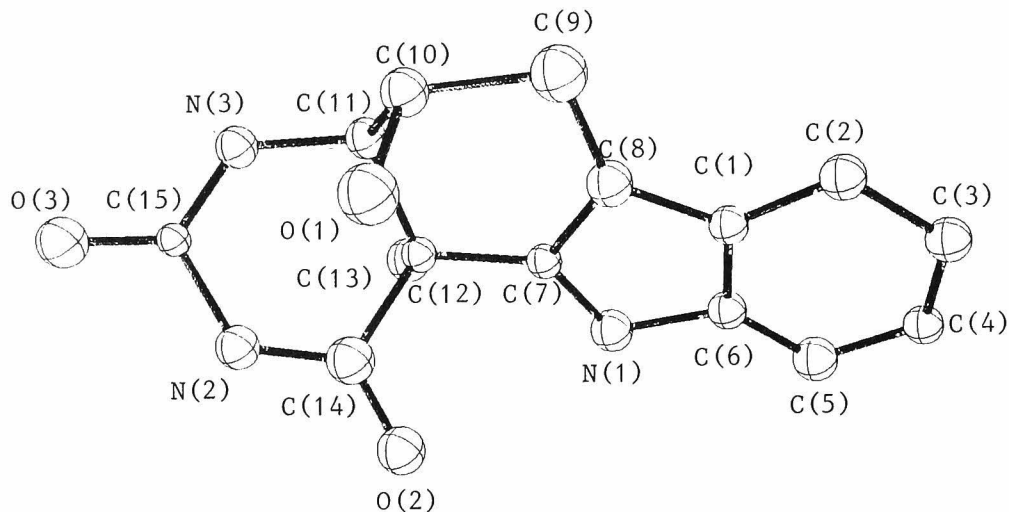


Figure 1. HPLC profiles of the photolysate (acetonitrile-water (20:80), 4 mL/min)

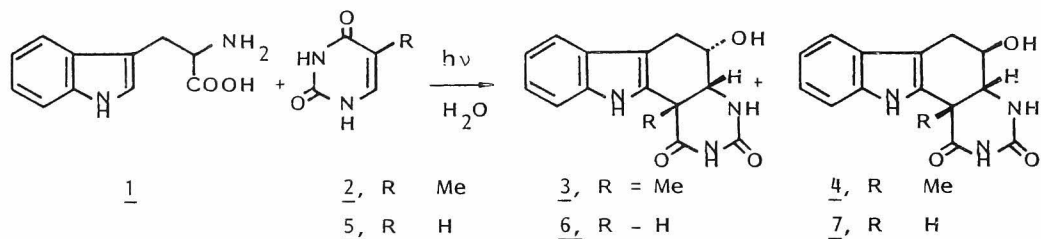
HPLC analysis as monitored at 254 nm revealed the presence of two products except for the starting materials, tryptophan and thymine. The eluate was evaporated and the residue was subjected to preparative TLC (silica gel, *n*-butanol-water (84:16)) to give ninhydrin negative photoproducts. These were further purified by preparative HPLC to give pure photoproducts 3 (15%) and 4 (14%). The structure of 3 shown in Scheme 1, was assigned by X-ray crystallographic analysis. The molecular structure of 3 is shown in Figure 2. The structure of 4 was assigned as 4 on the basis of its spectral data. Chromatographic behaviors and UV spectra of 3 and 4 are identical with those of photoproducts reported earlier by Reeve and Hopkins.⁶

Photoexcited tryptophan reacts with uracil (5) in a similar fashion to produce 6 (15%) and 7 (15%). The structures of 6 and 7

Figure 2. A Perspective Drawing of 3



Scheme 1.



were characterized by ^1H NMR and ^{13}C NMR spectroscopy. Assignment of the ^{13}C NMR signals is summarized in Table 1. These photoproducts (3, 4, 6 and 7) are presumed to be derived from the Diels-Alder type adducts between indole-3-acetaldehyde and thymine. In fact, irradiation of indole-3-acetaldehyde (8) with thymine (2) gave 3 (20%) and 4 (20%) under the condition. Similarly, photoreaction of 8 with uracil (5) provided 6 and 7. Therefore, this photoreaction is thought to proceed via indole-3-acetaldehyde (8). A reasonable mechanism for the formation of 3 and 4 is shown in Scheme 2.

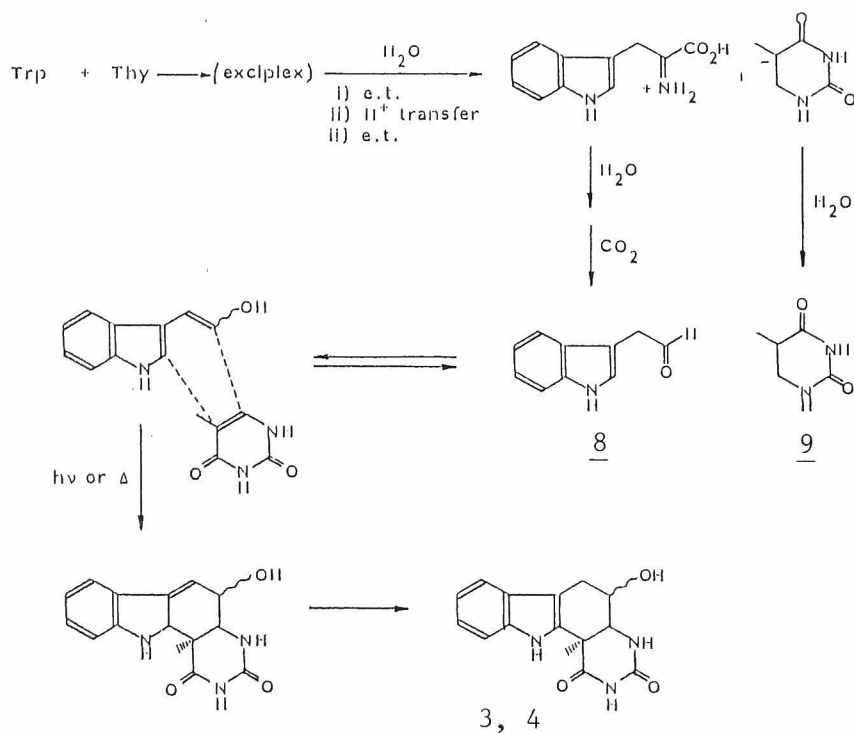
Table 1. ^{13}C NMR Chemical Shifts (δ , DMSO- d_6) for photoproducts 3, 4, 6 and 7.

atom	<u>3</u>	<u>4</u>	<u>6</u>	<u>7</u>
C - 1	132.4	132.0	127.7	127.1
C - 2	118.1	118.3	117.7	118.1
C - 3	121.2	121.5	120.8	121.6
C - 4	118.5	118.7	118.2	118.9
C - 5	111.5	111.5	111.3	111.7
C - 6	137.2	137.4	136.8	137.1
C - 7	126.7	126.0	126.6	126.6
C - 8	105.9	105.9	106.0	106.8
C - 9	27.9	29.4	27.6	28.2
C - 10	68.2	67.6	68.1	66.8
C - 11	58.9	60.7	51.9	52.6
C - 12	40.2	43.1	37.1	39.2
C - 13	24.4	23.0	-	-
C - 14	173.4	173.5	169.7	170.1
C - 15	153.8	152.4	153.7	152.8

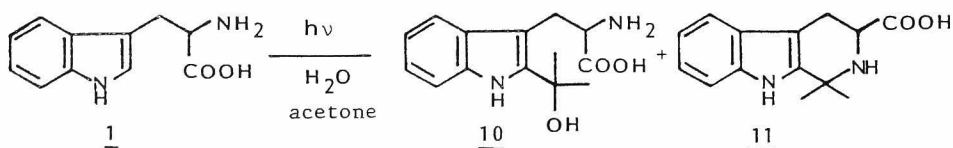
The first step may be a one-electron transfer occurring from photoexcited tryptophan to thymine. After multistep reactions including proton transfer and hydrogen transfer, indole-3-acetaldehyde (8) and dihydrothymine (9) are produced from tryptophan and thymine, respectively. This aldehyde (8) after enolization photochemically or thermally reacts with another molecule of thymine to produce 9, which is isomerized to 3 or 4.

In contrast to direct irradiation leading to 3 and 4, acetone sensitized irradiation of 1 in the presence of 2 in aqueous solution never produced 3 and 4 but gave tryptophan-acetone adducts 10 and 11 probably via an electron-transfer process (Scheme 3), indicating that the lowest triplet state of tryptophan is not responsible for the

Scheme 2.



Scheme 3.



photoreaction with thymine. Under the irradiation condition, 10 was readily converted to 11. The result indicates that 10 is the precursor of 11. The structures of 10 and 11 were deduced from the spectral data. 11 was also identified by comparison of the ^1H NMR spectrum with that of the authentic sample prepared independently.

Experimental

Melting points are uncorrected. Elemental analysis were performed at the Analytical Center of Kyoto University. Ultraviolet spectra were recorded with a Shimadzu UV-200 spectrophotometer. ^1H NMR spectra were measured on a Varian HA-100 or a JEOL JNM-GX 400 spectrometer using Me_4Si as the internal reference. Carbon-13 magnetic resonance spectra were recorded with a Varian FT-80A spectrometer. High-performance LC (HPLC) was performed on a Waters ALC/GPC 204 model equipped with a 254 nm or a 280 nm fixed wavelength detector. Reverse-phase μ -Bondapak C_{18} and Nucleosil 7C_{18} (1 x 25 cm) column were used for analytical and preparative HPLC, respectively. Irradiations were carried out by using a 10 W low-pressure mercury lamp (method A) or a 400 W high-pressure mercury lamp (method B). Vycor filters were used to cut off wavelength $\lambda < 240$ nm in all cases. Indole-3-acetaldehyde sodium bisulfite and 2,3,4,9-tetrahydro-1,1-dimethyl-1H-pyrido[3,4-*b*]indole (11) were prepared according to the published procedures.⁷ (11): ^1H NMR (D_2O) δ 1.59 (s, 3 H), 1.77 (s, 3 H), 2.91 (dd, 1 H, $J = 16, 12$ Hz), 3.30 (dd, 1 H, $J = 16, 5.5$ Hz), 4.02 (dd, 1 H, $J = 12, 5.5$ Hz), 6.96-7.60 (m, 4 H).

Irradiation of Tryptophan (1) with Thymine (2). A solution of 1 (204 mg, 1 mmol) and 2 (25 mg, 0.2 mmol) in distilled water (200 mL) was irradiated with a 10 W low-pressure mercury lamp under standard conditions (method A) for 12 h. After removal of the solvent, the residue was subjected to preparative TLC with *n*-butanol-water (84:16). The fraction of R_f 0.9-0.85 was separated to give the mixture of 3 and 4. The mixture was subjected to preparative HPLC (1 x 25 cm, Nucleosil 7C_{18} column, acetonitrile-water (1 : 4)) to give pure 3 (15%) and 4 (14%). 7H-4,6-dioxypyrimidino[5,6-*a*]1,2,2a β , 6a-tetrahydro-6a β -methyl-2 α -hydroxycarbazole (3): mp > 250 °C; UV (MeOH) 292 nm ($\log \epsilon$ 3.85), 283 (3.94); ^1H NMR ($\text{DMSO}-d_6$) δ 1.61 (s, 3 H), 2.76 (dd, 1 H, $J = 12, 4$ Hz), 2.82 (dd, 1 H, $J = 12, 4$ Hz), 3.45 (m, 1 H), 4.13 (m, 1 H), 5.14 (d, 1 H, $J = 4$ Hz, OH), 6.94 (dd,

1 H, J = 9, 7 Hz), 7.04 (dd, 1 H, J = 8, 7 Hz), 7.33 (d, 1 H, J = 8 Hz), 7.36 (d, 1 H, J = 9 Hz), 7.46 (s, 1 H, NH), 9.88 (brs, 1 H, NH), 10.88 (s, 1 H, NH); Anal. Calcd. for $C_{15}H_{15}N_3O_3 \cdot CH_3OH$: C; 60.55, H; 6.04, N; 13.24. Found: C; 60.79, H; 5.89; N; 13.46. 7H-4,6-dioxypyrimidino-[5,6- α]1,2,2a β ,6a-tetrahydro-6a β -methyl-2 β -hydroxycarbazole (4): mp > 250 °C; 1H NMR (DMSO- d_6) δ 1.64 (s, 3 H), 3.04 (dd, 1 H, J = 15, 5 Hz), 3.17 (dd, 1 H, J = 10, 5 Hz), 3.22 (dd, 1 H, J = 15, 5 Hz), 3.74 (m, 1 H), 5.48 (m, 1 H, OH), 6.95 (t, 1 H, J = 8 Hz), 7.06 (t, 1 H, J = 7 Hz), 7.36 (dd, 2 H, J = 8, 8 Hz), 7.89 (d, 1 H, J = 5 Hz, NH), 10.15 (br s, 1 H, NH), 11.07 (s, 1 H, NH). Exact mass. Found: 327.1239. Calcd. for $C_{17}H_{17}N_3O_4$ (monoacetate): 327.1219.

Irradiation of 1 with uracil (5). A solution of 1 (204 mg, 1 mmol) and 5 (22 mg, 0.2 mmol) in distilled water (200 mL) was irradiated with a 400 W high-pressure mercury lamp (method B) for 6 h. Photolysate was extracted with ethyl acetate (3 x 200 mL), and the organic layer was separated, dried and concentrated in vacuo to give the mixture of 6 and 7. The residue was subjected to preparative HPLC (acetonitrile-water (20 : 80)) to give pure 6 (15%) and 7 (15%). 7H-4,6-dioxypyrimidino[5,6- α]1,2,2a β ,6a β -tetrahydro- α -hydroxycarbazole (6): 1H NMR (DMSO- d_6) δ 2.79 (m, 2 H), 3.81 (m, 1 H), 4.01 (d, 1 H, J = 7 Hz), 4.10 (br d, 1 H), 5.13 (d, 1 H, J = 4 Hz, OH), 6.94 (dd, 1 H, J = 8, 7 Hz), 7.03 (dd, 1 H, J = 8, 7 Hz), 7.34 (d, 1 H, J = 8 Hz), 7.36 (d, 1 H, J = 8 Hz), 7.50 (brs, 1 H, NH), 9.91 (s, 1 H, NH), 10.76 (s, 1 H, NH). Exact mass. Found: 271.0895. Calcd. for $C_{14}H_{13}N_3O_3$: 271.0958. 7H-4,6-dioxypyrimidino[5,6- α]1,2,2a β ,6a β -tetrahydro- β -hydroxycarbazole (7): 1H NMR (DMSO- d_6) δ 2.53 (m, 1 H), 3.01 (dd, 1 H, J = 17, 6 Hz), 3.67 (m, 1 H), 3.89 (m, 1 H), 4.83 (d, 1 H, J = 6 Hz), 5.37 (d, 1 H, J = 5 Hz, OH), 6.95 (dd, 1 H, J = 8, 8 Hz), 7.04 (dd, 1 H, J = 8, 8 Hz), 7.34 (d, 1 H, J = 8 Hz), 7.37 (d, 1 H, J = 8 Hz), 7.82 (br s, 1 H, NH), 10.22 (s, 1 H, NH), 10.77 (s, 1 H, NH).

Irradiation of Indole-3-acetaldehyde (8) with Thymine (2).

A solution of 8 (263 mg, 1 mmol) and 2 (25 mg, 0.2 mmol) in distilled water (200 mL) was adjusted to pH 7.0 with 1 N NaOH and was irradi-

ated with a 400 W high-pressure mercury lamp through Vycor filter for 5 h. HPLC analysis of the reaction mixture revealed the formation of 3 (20%) and 4 (20%).

Irradiation of Indole-3-acetaldehyde (8) with Uracil (5).

A solution of 8 (263 mg, 1 mmol) and 5 (22 mg, 0.2 mmol) in distilled water (200 mL) was adjusted to pH 7.0 with 1 N NaOH and was irradiated with a 400 W high-pressure mercury lamp through Vycor filter for 5 h. HPLC analysis of the photolysate indicated the formation of 6 (20%) and 7 (20%).

Irradiation of Tryptophan (1) in Acetone-Water.

A solution of 1 (408 mg, 2 mmol) in acetone-water (200 mL, (1:1)) was irradiated with a high pressure mercury lamp through Pyrex filter under nitrogen atmosphere for 3 h. HPLC analysis (acetonitrile-water, (20:80)) showed the formation of photoadducts 10 and 11 with unreacted starting compound 1. After removal of the solvent the residue was subjected to preparative HPLC to give 10 (24%), 2,3,4,9-tetrahydro-1,1-dimethyl-1H-pyrido[3,4-*b*]indole (11, 17%).

¹H NMR and chromatographic behaviours (TLC, HPLC) of the product 11 were identical with those of the authentic sample prepared independently. 2(1-Hydroxyisopropyl)tryptophan (10): Viscous oil ¹H NMR (D₂O) δ 1.60 (s, 3 H), 1.62 (s, 3 H), 3.34 (dd, 1 H, J = 15, 9 Hz), 3.56 (dd, 1 H, J = 15, 5.5 Hz), 3.92 (dd, 1 H, J = 9, 5.5 Hz), 6.94-7.72 (m, 4 H); ¹³C NMR (D₂O) δ 175.4, 143.6, 135.6, 129.1, 122.8, 120.3, 119.0, 112.3, 103.8, 71.9, 55.7, 31.2, 30.8, 26.9. Exact mass. Found: 276.1423. Calcd. for C₁₅H₂₀N₂O₃ (methy ester) 276.1474.

X-ray Analysis.

The compound 3 was crystallized from acetic acid as colorless plates. The crystal data are as follows: C₁₅H₁₅N₃O₃, M = 285.3, orthorhombic, space group P2₁2₁2₁, a = 29.49, b = 7.84, c = 6.96 Å, Z = 4, V = 1609 Å³. Diffraction data were measured on a microcomputer-controlled four-circle diffractometer.⁷ The unit cell dimensions were derived from a least-squares fit to the observed values of $\pm \theta$ for 11 reflections. Of 1193 independent reflections measured up to $2\theta = 110^\circ$ by a $\theta - 2\theta$ scan technique using CuK α

radiation, 980 reflections whose intensities were greater than 1.0. $\sigma(I)$ were used for subsequent calculations. A periodically monitored reflections showed no significant change in intensity. The intensities were corrected only for the Lorentz and polarization factors. The quality of the intensity data was rather poor due to the small size (ca. 0.1 x 0.2 x 0.02 mm) of the crystal.

The structure was solved by the direct method.⁸ The E-map based on the phase set of the highest combined figure of merit gave a fragment of the molecule. The remaining non-hydrogen atoms were located in the successive Fourier synthesis. The structure was refined by the block-diagonal least-squares method.⁹ Several additional peaks were found along the two-fold screw axis along the c-axis in the Fourier synthesis. They must be disordered solvent, but it was not possible to assign what they were. The current R-value is 0.22 for 980 reflections. Final atomic coordinates of 3 for non-hydrogen atoms are listed in Table 2.

I am indebted to Professor Yukiteru Katsube, Osaka University and Dr. Keiichi Fukuyama, Tottori University for the X-ray crystallographic analysis.

Table 1. Final atomic coordinates of 3 for non-hydrogen atoms.

C(1)	0.25069	0.28018	0.26402
C(2)	0.29087	0.34246	0.34353
C(3)	0.33074	0.26140	0.29919
C(4)	0.33217	0.12740	0.16853
C(5)	0.29325	0.06382	0.08355
C(6)	0.25193	0.15030	0.13661
C(7)	0.17956	0.23082	0.16361
C(8)	0.20344	0.33782	0.27820
C(9)	0.18190	0.48636	0.38782
C(10)	0.12997	0.45811	0.39681
C(11)	0.11010	0.42418	0.22672
C(12)	0.12988	0.24132	0.13411
C(13)	0.11971	0.23566	-0.06487
C(14)	0.10682	0.06646	0.19833
C(15)	0.03696	0.25883	0.24307
N(1)	0.20805	0.11345	0.07234
N(2)	0.05942	0.08141	0.23869
N(3)	0.06041	0.41717	0.22808
O(1)	0.12016	0.29789	0.50947
O(2)	0.12290	-0.09354	0.19985
O(3)	-0.00496	0.25257	0.25362

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CHAPTER 8

A Novel Photochemical Hydrogen Exchange Reaction of Tryptophan at C-4 Position

ABSTRACT

Irradiation of tryptophan in D_2O resulted in a rapid incorporation of the deuterium into C-4 position of the indole nucleus in a highly regiospecific fashion. Irradiation of Trp-4- d_1 in water gave rise to Trp. Both reactions proceeded with remarkably high quantum efficiency. These observations indicate that this type of photochemical hydrogen exchange reaction occurring in normal light water is one of the major modes of nonradiative decay of the singlet excited state of Trp. Specific labeling of L-tryptophyl-L-tyrosine was also discussed.

INTRODUCTION

Numerous biophysical and photophysical studies have devoted to the photochemistry and fluorescence properties of tryptophan (Trp), the parent indoles, Trp-containing oligopeptides and proteins, owing to the utility of Trp fluorescence as intrinsic probe in the structure and function of proteins and enzymes.^{1,2,3} Particularly intriguing is the mechanism of an enhancement of the fluorescence quantum yield of Trp in D₂O.² Despite extensive efforts the mechanism of nonradiative deactivation processes of the excited singlet state of Trp is not well understood though some interpretations including a proton-transfer process from the α -ammonium group of the side chain to the singlet excited indole ring have been proposed for the quenching of the fluorescence of Trp.³ We report herein a remarkably efficient and highly selective photoexchange reaction of the C-4 hydrogen of Trp with the deuterium of solvent D₂O which has been overlooked for a number of years. This photochemical hydrogen exchange reaction of Trp occurring in normal light water giving rise to original Trp molecule as a photoproduct, *i.e. photoreaction of Trp without photoproduct*, is demonstrated to be a one of the major modes of nonradiative decay of the singlet excited state of Trp. Furthermore, we show that this novel type of photoexchange reaction in D₂O can be widely applicable to the specific labeling of Trp, other indoles and Trp-containing peptides.⁴

RESULTS AND DISCUSSION

External irradiation of a degassed solution of Trp (10 mM) in unbuffered D₂O (99.9% D) in a 5 mm ϕ NMR sample tube with Vycor filtered (>254 nm) light from a 400 W high pressure mercury lamp resulted in a rapid incorporation of the deuterium from D₂O into the indole nucleus as evidenced by ¹H NMR. Figure 1 illustrates the progress of the deuterium incorporation as monitored by 400 MHz ¹H NMR. Deuterium was incorporated up to 92% into the 4-position

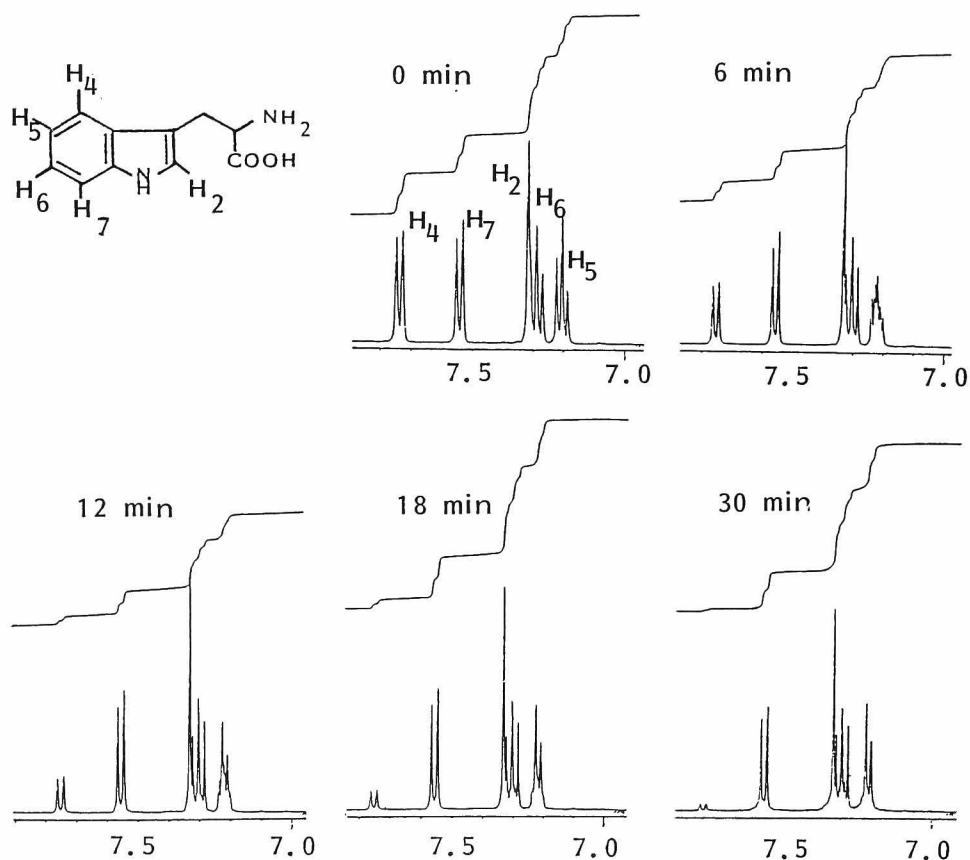


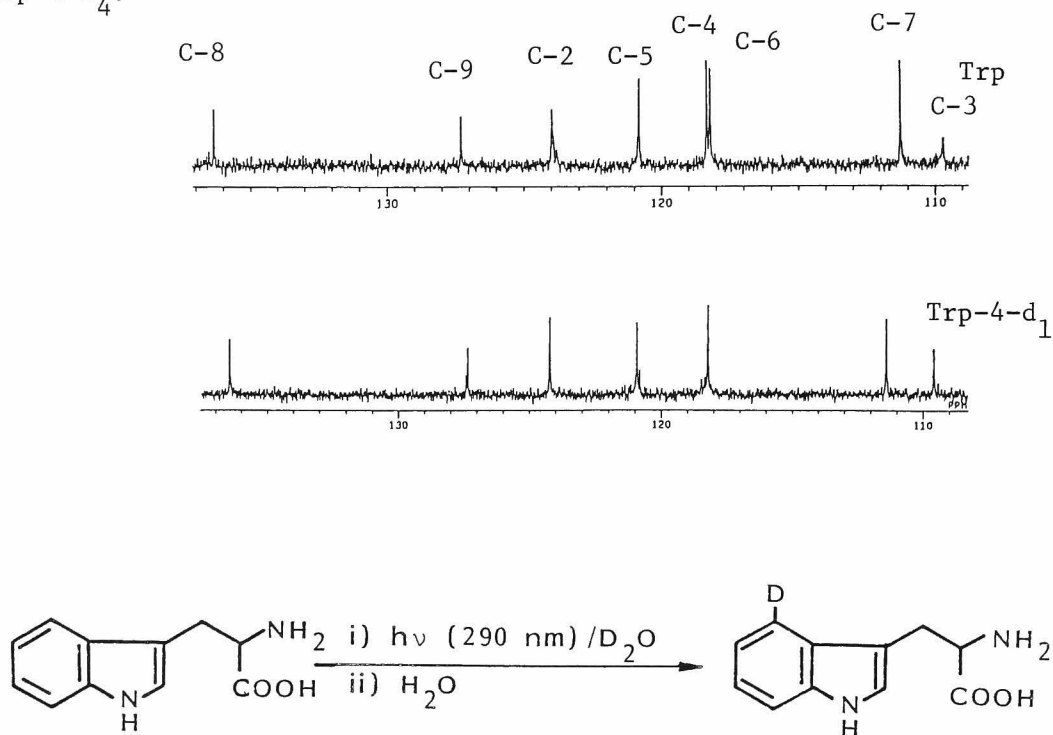
Figure 1. Progressive NMR spectral change induced by irradiation of tryptophan in D_2O .

of Trp with high regioselectivity within 30 min irradiation. Assignment of the deuterated position of the indole ring was made on the basis of 1H - and ^{13}C NMR (Figure 2), and the deuterium content was determined by careful integration of the signal at 7.75 by 400 MHz 1H NMR and by mass spectrometry after treatment with water.

Quantum yield of the deuterium incorporation from D_2O was determined as 0.18 in a optical bench fitted with a monochromator at the excitation wavelength of 290 ± 9.5 nm by employing potassium ferrioxalate actinometry. No decrease in quantum yield was observed with saturating concentrations of N_2O in D_2O ,

indicating that photoionization of Trp to hydrated electrons and $\text{Trp}^{\cdot+}$ is not responsible for the photoexchange reaction.

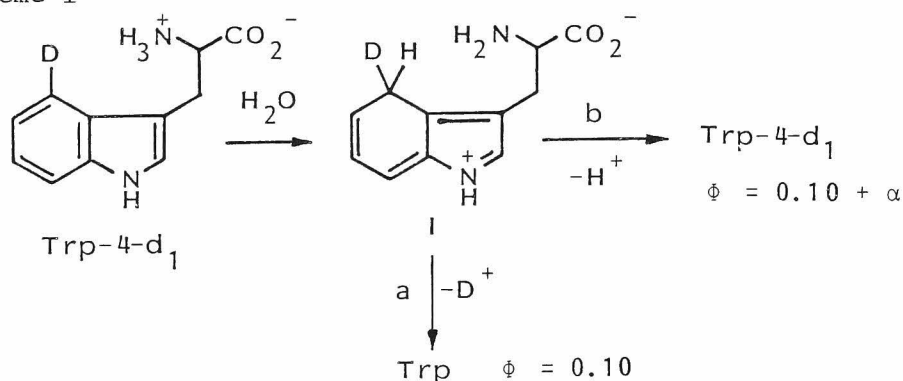
Figure 2. ^{13}C NMR spectra of aromatic carbons for Trp and Trp-4-d₄.



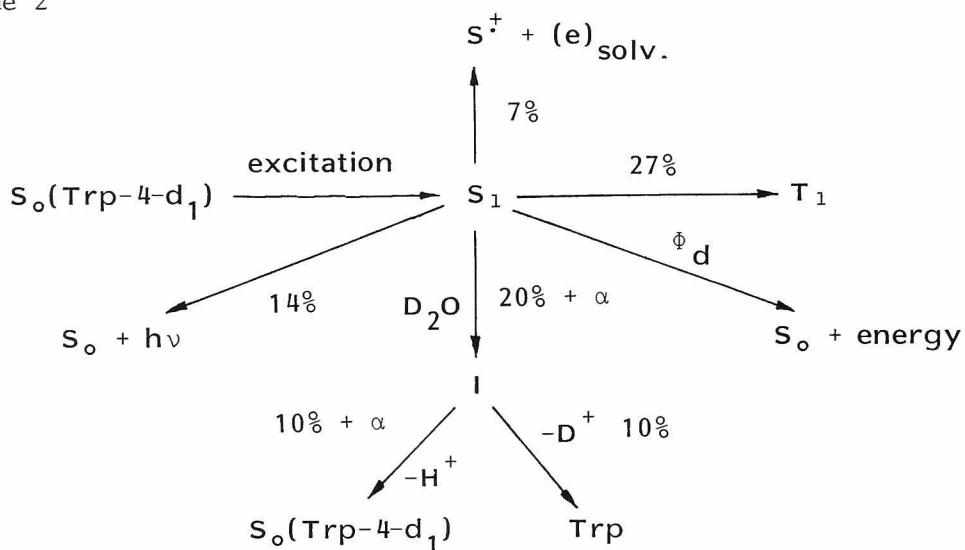
Irradiation of 4-deuterated Trp (Trp-4-d₁) containing 98% D in 50 mM phosphate buffer at pH 7 resulted in loss of the C-4 deuterium into the solvent with the quantum yield of 0.10. This implies that a similar photochemical hydrogen exchange reaction is efficiently occurring at the C-4 position upon irradiation of Trp in water at neutral pH but this reaction gives no photoproduct. The efficiency of the photoexchange reaction of Trp-4-d₁ is a function of the solution pH. At below pH 7 where the α -amino group of the side chain is protonated, the photoexchange reaction

of Trp-4-d₁ proceeds efficiently to result in a loss of deuterium, whereas at pH 10 the concentration of proton is insufficiently to result in a lower quantum yield 0.04. The pH dependency parallels the reported variations in fluorescence quantum yield of Trp. Based on these experimental observations we propose a following mechanism for the deuterium loss of Trp-4-d₁ at a neutral pH as shown in Scheme 1. An intramolecular proton-transfer from the α -ammonium group to the 4-position of the singlet excited indole ring would occur as a first step to give the intermediate 1 which then loses a deuteron (path a) or a proton (path b) to give non-deuterated Trp (0.10) and original Trp-4-d₁. Unfortunately, quantum yield of the latter process (path b) cannot be obtained experimentally. However, it is obvious judging from the expected isotope effect for the proton loss from the intermediate 1 that the quantum efficiency may well exceed 0.10. Thus, the quantum yields for all processes occurring from the excited singlet state of Trp-4-d₁ at pH 7 are shown in Scheme 2, provides that the quantum yields of intersystem crossing and monophotonic photoionization for Trp-4-d₁ are the same as those reported for Trp. As apparent from Scheme 2 the extent of the photoexchange reaction with water leading to non-deuterated Trp

Scheme 1



Scheme 2



is estimated to be greater than 38% of the sum of all radiationless deactivation processes (0.52) except for intersystem crossing and photoionization. Thus our preliminary results indicate that the reaction of singlet excited Trp with water leading to the exchange of C-4 hydrogen plays a major role in nonradiative deactivation of the singlet Trp, although the details should await further precise study. These results also suggest that this type of photoexchange reaction is primarily responsible for the enhancement of the fluorescence intensity of Trp in D₂O

Irradiation of other indoles in D₂O or methanol-d₁ containing D₂O under similar conditions resulted in the deuterium incorporation at C-4, C-2 and C-7 positions. For example, irradiation of tryptamine hydrochloride in D₂O resulted a deuteration at C-4 (59%), C-2 (19%) and C-7 (28%). Irradiation of 3-indolepropionic acid and its N-methyl derivative produced the corresponding 4- and 2-deuterated products. Irradiation of L-tryptophyl-L-tyrosine in unbuffered D₂O resulted in a selective deuteration of the C-4

position of Trp moiety without incorporating into the tyrosine unit as shown in Figure 3. Thus, the method may be used for specific labeling of the C-4 position of Trp residues in a protein with deuterium or tritium.

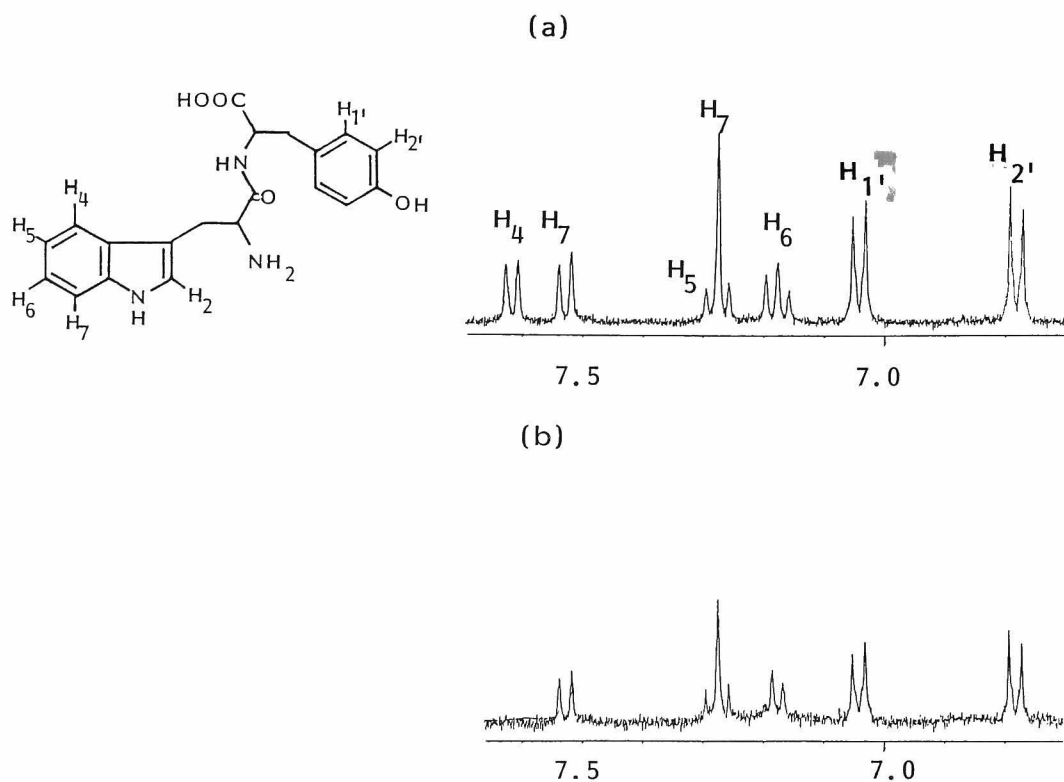


Figure 3. 400 MHz ^1H NMR of L-tryptophyl-L-tyrosine in D_2O :
(a) before irradiation, (b) after 30 min irradiation.

EXPERIMENTAL

Ultraviolet spectra were recorded with a Shimadzu UV-200 spectrophotometer. ^1H - and ^{13}C NMR spectra were recorded with a JEOL JNM-GX400 spectrometer using 3-(trimethylsilyl)propionic acid- d_4 -sodium salt (TSP) as the internal reference. Mass spectra were recorded with a JEOL-JMS-DX300 spectrometer. High-performance LC (HPLC) was performed on a Waters ALC/GPC 204 model equipped with a 254 nm fixed wavelength detector. Reverse-phase μ Bondapak C_{18} and Nucleosil 7C $_{18}$ (1 x 25 cm) columns were used for analytical and preparative HPLC, respectively. Irradiation was carried out by using a 400 W high-pressure mercury lamp. Vycor filter was used to cut off wavelengths of $\lambda < 240$ nm in all cases. L-Tryptophyl-L-tyrosine was purchased from Sigma and used without purification.

Irradiation of Tryptophan in D_2O . A solution of tryptophan (1 mg, 0.005 mmol) in D_2O (0.5 mL) in a 5 mm ϕ NMR sample tube (Pyrex, Wilmad) was purged with nitrogen and was irradiated with a 400 W high-pressure mercury lamp through Vycor filter externally. 400 MHz ^1H NMR spectra were measured periodically. The deuterium content was determined by integration of the signal at 7.75 ppm. The results are shown in Figure 1 and Table I. In a preparative run, a solution of tryptophan (20 mg, 0.1 mmol) in D_2O (5 mL) was irradiated as described above for 4 h. Photolysate was subjected to preparative HPLC (acetonitrile-water (15:85), 10 mL/min) to give 4-deuterated tryptophan containing 98% D (18 mg, 90%).

4-Deuterated Tryptophan (Trp-4- d_1). UV (H_2O) 276 nm ($\log \epsilon$ 3.75); ^1H NMR (D_2O) δ 3.31 (dd, 1 H, $J = 15, 8$ Hz), 3.48 (dd, 1 H, $J = 15, 5$ Hz), 4.06 (dd, 1 H, $J = 8, 5$ Hz), 7.20 (d, 1 H, $J = 8$ Hz), 7.29 (dd, 1 H, $J = 8, 7$ Hz), 7.32 (s, 1 H), 7.54 (d, 1 H, $J = 8$ Hz); ^{13}C NMR ($\text{DMSO}-\text{d}_6$) δ 27.2, 54.9, 109.6, 111.4, 118.2, 120.8, 124.2, 127.3, 136.5, 170.7. Exact mass. Found: 219.1118. Calcd. for $\text{C}_{12}\text{H}_{13}\text{N}_2\text{O}_2\text{D}$ (methylester): 219.1118.

Irradiation of Tryptamine Hydrochloride in D_2O . A solution of

tryptamine hydrochloride (1 mg, 0.005 mmol) in D_2O (0.5 mL) in a NMR sample tube was purged with nitrogen and irradiated as described above for 30 min. After irradiation NMR spectrum was measured. The deuterium content of the indole ring protons were determined by integration of the proton signal at δ 7.32 (C-2), 7.70 (C-4), and 7.55 (C-7). The results are as follows: 17% D at C-2, 59% D at C-4, and 28% D at C-7.

Irradiation of 3-Indolepropionic Acid in D_2O . A solution of 3-indolepropionic acid (1 mg, 0.005 mmol) in D_2O (0.5 mL) in a NMR sample tube was purged with nitrogen and irradiated as described above for 30 min. After irradiation NMR spectrum was measured. The deuterium contents at C-2, C-4 and C-7 are 26%, 28% and 8%, respectively.

Irradiation of N-Methyl-3-Indolepropionic Acid in D_2O . A solution of N-methyl-3-indolepropionic acid (1 mg, 0.005 mmol) in D_2O (0.5 mL) in a NMR sample tube was purged with nitrogen and irradiated as described above for 30 min. After irradiation NMR spectrum was measured. The deuterium contents are as follows: 31% D at C-2 and 38% D at C-4.

Irradiation of L-Tryptophyl-L-Tyrosine in D_2O . A solution of L-tryptophyl-L-tyrosine (1 mg, 0.003 mmol) in D_2O (0.5 mL) in a NMR sample tube was purged with nitrogen and irradiated as described above for 30 min. After irradiation NMR spectrum was measured. The results are shown in Figure 3.

Quantum-Yield Measurement. Quantum-yield measurements were carried out at room temperature in a optical bench fitted with a monochromator at the excitation wavelength of 290 ± 10 nm by using potassium ferrioxalate actinometry. Sample solutions contained 1.2 mM of tryptophan in D_2O . Under the condition tryptophan absorbs more than 99% of the incident light. The irradiation was stopped at less than 20% conversion of the exchange reaction. The deuterium content of the indole ring was determined by NMR spectroscopy.

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- Chapter 1 Saito, I.; Sugiyama, H.; Furukawa, N.; Matsuura, T.
Tetrahedron Lett. 1981, 22, 3265.
- Chapter 2 Saito, I.; Sugiyama, H.; Ito, S.; Furukawa, N.;
Matsuura, T. J. Am. Chem. Soc. 1981, 103, 1598.
- Chapter 3 Saito, I.; Sugiyama, H.; Matsuura, T. J. Am. Chem.
Soc. 1983, 105, 956.
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Nucleic Acids Res. 1981, Sp. No 10, 61.
- Chapter 4 Saito, I.; Sugiyama, H.; Matsuura, T.; Ueda, K.;
Komano, T. submitted to Nucleic Acids Res.
Sugiyama, H.; Saito, I.; Matsuura, T.; Ueda, K.;
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- Chapter 5 Saito, I.; Sugiyama, H.; Matsuura, T. J. Am. Chem.
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- Chapter 6 Matsuura, T.; Saito, I.; Ito, S.; Sugiyama, H.;
Shinmura, T. Pure Appl. Chem. 1980, 52, 2705.
- Chapter 7 to be submitted.
- Chapter 8 Saito, I.; Sugiyama, H.; Matsuura, T. submitted to
J. Am. Chem. Soc.

The following papers, which are not included in this thesis, have been published by the author et al.

- (1) Saito, I.; Tamoto, A.; Katsumura, A.; Sugiyama, H.; Matsuura, T. Chemistry Letters 1978, 127.
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