apparent dissociation constants of Ca^{2+}–dihydroxy bile salt complexes are dependent on the position and orientation of the hydroxy groups. It is possible for these bile salts that particular structural configurations favor complexation of Ca^{2+} between ionized side chains and the hydroxy groups. On the other hand, the difference in Ca^{2+} binding to dihydroxy vs. trihydroxy bile salts may be influenced by factors such as weaker metal ion binding to micelles, the critical micellar concentration, and the solubility product of the bile salt–metal ion complex (Jones, C. A.; Hofmann, A. F.; Mysels, K. J.; Roda, A., unpublished data). Therefore, our conclusions about the absence of Ca^{2+} chelation to hydroxy groups for the trihydroxy bile salt systems are not necessarily transferable to the dihydroxy bile salts.

The biological implication of our data is that species which have a large excess of taurocholate will be less able to lower free Ca^{2+} activity than those with glycocholate. This may be a reason why dogs, which have mostly taurocholate in the bile, have evolved with a low cholesterol saturation index, whereas omnivores such as man and hamsters tolerate cholesterol supersaturation in the bile. The ratio of glycocholate to taurocholate in humans is about 3:1, and the concentration of total calcium in the bile is 3–5 mM. Since cholesterol gallstone formation in man requires other initiating factors besides a cholesterol supersaturated bile, such as a precipitated calcium nidus, the role of bile salts in suppressing the effects of free Ca^{2+}, in particular glycocholate, which has a calcium dissociation constant of 9.5 mM, may have had a major evolutionary influence on the physiology and chemistry of the biliary system.

Acknowledgment. This study was supported in part from funds derived from Grant AM-25511 from the National Institute of Arthritis, Metabolism, Digestive and Kidney Diseases and from funds for a pilot project from the Cystic Fibrosis Foundation. The NMR instrumentation used was supported in part by NIH Grant CA-13148 to the NMR Core Facility of the UAB Comprehensive Cancer Center. We thank Dr. Alan F. Hofmann, University of California, San Diego, for providing us, prior to publication, with a copy of his results.

Registry No. GC, 475-31-0; TC, 81-24-3; Ca, 7440-70-2; Na, 7440-23-5; Dy, 7429-91-6.

(37) Abbreviations: GC, 3α,7α,12α-trihydroxy-5β-cholan-24-oylglycine (glycocholate); TC, 3α,7α,12α-trihydroxy-5β-cholan-24-oyltaurine (taurocholate); NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; pH*, uncorrected pH measured in D_2O; Gly, glycine; Tau, taurine.

Phototransformed Bleomycin Antibiotics. Structure and DNA Cleavage Activity†

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Abstract: The light irradiation of copper(II)–bleomycin and its bithiazole model compound, methyl 2′-methyl-2,4′-bithiazole-4-carboxylate, induced isomerization of the 2,4′-bithiazole ring system to 4,4′-bithiazole. Phototransformed bleomycin and the model compound were isolated in high yields by using high-performance liquid chromatography. The electronic absorption, fluorescence emission, and ^1H and ^{13}C NMR data of the new photoproducts were compared with those of the original bleomycin and the model compound. Of special interest is the fact that the phototransformed bleomycin showed the nucleotide sequence cleavage mode to be essentially the same as that of the parent bleomycin. The present results provide valuable information not only for the photoreactivity of bleomycin antibiotics but also for the DNA recognition of the bithiazole group.

Bleomycin antibiotics have been clinically employed for the treatment of squamous cell carcinoma and malignant lymphoma. The oxidative DNA strand scission mediated by certain metallobleomycins is believed to be responsible for their therapeutic effects.[1] Usually, the DNA cleavage of bleomycin is thought to result from metal chelation and oxygen activation by the β-aminoalanine–pyrimidine–β-hydroxyhistidine moiety and DNA interaction by the bithiazole–terminal amine portion.[2] Recently, bleomycin antibiotics are reported to be photosensitive. We found that the bleomycin–Fe(III) complex produced prominent DNA strand scission by photoirradiation as well as by the presence of reductants or hydrogen peroxide.[3] By contrast, the bleomycin–Co(III) complex significantly degraded isolated DNA only by light irradiation.[4,5] While the UV irradiation of bleomycin alone by a medium-pressure mercury lamp or a xenon 300-W lamp induced the decrease of the absorption band at 290 nm due to the bithiazole chromophore,[6,7] details of the photoreaction of bleomycin antibiotics are entirely unknown. It is of particular importance to clarify the photolability of bleomycin in association with its DNA cleavage activity. Moreover, the role of the bithiazole group is still questionable in the bleomycin action on DNA.

Herein, we first demonstrate a transformation of the bithiazole group by light irradiation of the bleomycin–Cu(II) complex. Of interest is the fact that the nucleotide sequence cleavage mode of the phototransformed bleomycin was remarkably similar to that of the original bleomycin. The present results provide useful

† This paper is dedicated to Prof. Morio Ikehara for the occasion of his retirement from Osaka University in March, 1986.

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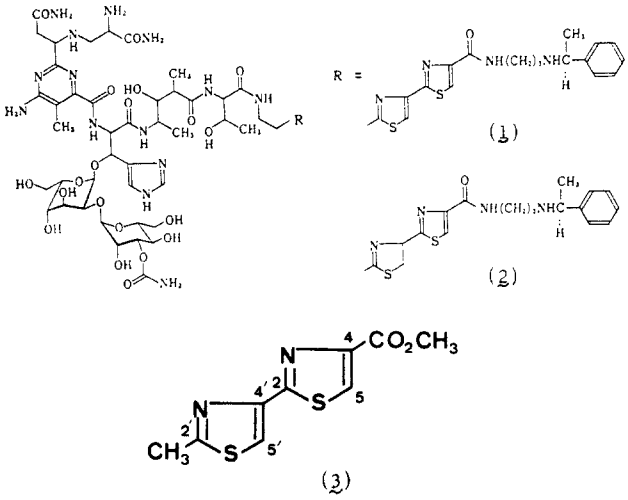

Figure 1. Chemical structures of bleomycin antibiotics and the bithiazole model compound: peplomycin (PEM) (**1**), phleomycin (**2**), and methyl 2′-methyl-2,4′-bithiazole-4-carboxylate (BT) (**3**).

information for the DNA recognition by the bithiazole group as well as for the photoreactivity of bleomycin antibiotics. The structures of bleomycin antibiotics and the bithiazole model compound used in this study are given in Figure 1.

Experimental Section

Materials. Purified bleomycin antibiotics, peplomycin (PEM) and bleomycin A_2, were supplied by Nippon Kayaku Co. Phleomycin was provided from the Institute for Microbial Chemistry, Tokyo. The bithiazole model compound, methyl 2′-methyl-2,4′-bithiazole-4-carboxylate (BT), was prepared as described by Riordan and Sakai.[8] Milli Q reagent grade water was used throughout the experiments.

Methods. Photoirradiation was carried out with an ultraviolet 60-W transilluminator TM-36 or a 400-W high-pressure mercury lamp. High-performance liquid chromatography (HPLC) was performed on a Waters ALC/GPC 204 model equipped with a 254- or 214-nm fixed-wavelength detector. A reverse-phase Nova-PAK C_{18} column (8 mm × 15 cm) and a Nucleosil $7C_{18}$ column (10 mm × 25 cm) were used for analytical and preparative HPLC, respectively. Preparative thin-layer chromatography (TLC) was carried out on Merck 60 PF_{254} silica gel plates. Electronic absorption and fluorescence emission spectra were measured on a Hitachi 330 spectrophotometer and a Shimadzu RF-540 spectrofluorometer. Mass spectra were recorded with a JEOL-JMS-DX300 spectrometer. ^1H and ^{13}C NMR spectra were obtained by using a JEOL-JNM-GX400 spectrometer. The proton chemical shifts were given with respect to sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate (TSP) or tetramethylsilane as internal standard, and the ^{13}C chemical shifts were measured relative to the central peak of methanol-d_4 or chloroform-d_1 and converted to the tetramethylsilane scale (δ_{CD_3OD} 49.3 and δ_{CDCl_3} 76.9). X-band ESR spectra for the Cu(II) complexes of the original and phototransformed PEMs were measured at pH 7.5 and 77 K with a JES-FE-3X spectrometer.

Photoreaction of PEM (1). A solution containing PEM (300 μM) and $CuSO_4$ (330 μM) in 10 mM Tris–HCl buffer (pH 7.6, 15 mL) was irradiated at a distance of 5 cm from a 60-W transilluminator (302-nm light) through a Pyrex tube for 2.5 h. After the irradiation at ambient temperature (~35°), the reaction solution was evaporated to dryness under reduced pressure at 20 °C. The residue was subjected to HPLC using a Nova-PAK C_{18} column and eluted with the solvent (1% aqueous CH_3COONH_4–CH_3CN 84:16, 2.0 mL/min), and the fraction of a main peak (retention time, 18.2 min) was collected. After removal of the solvent in vacuo, 100 mM Na_2EDTA (2 mL) and 0.5 M HCl (50 μL) were added to the residue. The solution was passed through a Sep-PAK C_{18} column and washed successively with 100 mM Na_2EDTA (2 mL), 10 mM HCl (2 mL), and distilled water (3 mL). Elution with methanol (5 mL) followed by evaporation under reduced pressure gave metal-free phototransformed peplomycin (PEMH, **5**) which was homogeneous as determined by HPLC. The yield of the photoproduct PEMH was 53%. The major photoproduct of bleomycin A_2 was isolated in the same manner as described for phototransformed peplomycin, except for the HPLC experimental conditions (elution solvent, 1% aqueous ammonium acetate–water 9:1; flow rate, 1.5 mL/min; and retention time, 13.0 min).

Photoreaction of BT (3). A solution of BT (45 mg) in 250 mL of acetonitrile was irradiated at a distance of 5 cm from a 400-W high-pressure mercury lamp through a Pyrex filter for 1.5 h. After the irradiation at ambient temperature, the solvent was removed under reduced pressure and the residue was subjected to preparative silica gel TLC using methanol/chloroform (2.5:97.5). The eluate from the major band on the TLC plate was then subjected to preparative HPLC using methanol/water (47:53) as elution solvent. Collection of the peak with a retention time of 19 min followed by evaporation of the solvent gave the photoproduct BTH (**4**) as white powder (20 mg, 44% yield).

Determination of DNA Cleavage Activity. The activity of DNA strand scission for the original and phototransformed PEMs was determined on bacteriophage φX174 RF DNA by using gel electrophoresis of 0.8% agarose which contained ethidium bromide (0.5 μg/mL). A restriction fragment of 396 base pairs was obtained by digestion of pBR322 plasmid DNA with the restriction endonuclease *Hin*fI. After incubation with bacterial alkaline phosphatase, the 5′ ends were labeled with ^{32}P by treatment with bacteriophage T4 polynucleotide kinase and [γ-^{32}P]ATP. The DNA fragments of 5′-^{32}P singly end-labeled 327 and 301 base pairs were obtained by digestion of the doubly end-labeled 396 base pair fragment with *Hae*III and *Hha*I, respectively. The reaction sample containing the ^{32}P-labeled DNA piece and sonicated calf thymus carrier DNA (1 μg) was incubated with the iron(II) or cobalt(III) complex of PEM or PEMH in 20 mM Tris–HCl buffer (pH 7.5) at 37 °C for 2 min. After addition of 2-mercaptoethanol (1 mM), the reaction solution containing the DNA and PEM–Fe complex was incubated at 37 °C for 7 min, and then Na_2EDTA (final concentration, 20 mM) was added to stop the reaction. The reaction solution containing the DNA and the PEM–Co complex was irradiated at a distance of 3 cm from a 60-W transilluminator (302-nm light) through a Pyrex glass at 15 °C for 25 min. In all these cases, the DNAs were precipitated by addition of cold ethanol, and the DNA samples were subjected to electrophoresis together with the Maxam–Gilbert sequencing fragments[9] on a 10% polyacrylamide/7 M urea slab gel.

Results and Discussion

The 290-nm absorption band of the 1:1 PEM–Cu(II) complex was gradually decreased by light irradiation at neutral pH, showing two isosbestic points at 268 and 317 nm (Figure 2). HPLC analysis of the irradiation mixture revealed the formation of one major photoproduct together with a minor byproduct, and the analysis confirmed a substantial decrease of the starting Cu(II)–PEM (Figure 2). In contrast, the HPLC of the mixture obtained by irradiating PEM alone under the same condition was much more complicated. The Cu(II)-containing photoproduct (fraction 2) isolated by means of preparative HPLC yielded copper-free phototransformed peplomycin (PEMH) by treatment with Na_2EDTA. By combination of photoreaction and metal complexation, we first succeeded to isolate pure PEMH. It should be noted that the phototransformed PEM was considerably photostable, and PEMH did not revert back to the starting PEM by the UV irradiation. On the other hand, the light irradiation of the 1:1 phleomycin–Cu(II) complex showed no change of the 290-nm absorption band, and indeed the HPLC analysis gave no formation of photoproduct. Phleomycin differs from peplomycin in the bithiazole ring structure. One of the bithiazole rings is reduced in phleomycin (see Figure 1). Therefore, the present photoreaction appears to require the conjugation of bithiazole rings.

The electronic absorption and fluorescence emission of the original and phototransformed PEMs in methanol were as follows: PEM, λ_{max} 290 nm (log ε 4.11) and λ_{ems}^{max} 353 nm (strong, excitation at 290 nm); PEMH, λ_{max} 293 nm (log ε 3.78) and λ_{ems}^{max} 410 nm (moderate, excitation at 290 nm). In bleomycin antibiotics, the UV absorption at 290 nm and the fluorescence band at 350 nm are due to the bithiazole chromophore. The emission peak of PEMH at 410 nm is likely to originate partly from the 4-aminopyrimidine group of the compound, since the 4-aminopyrimidine group is known to fluoresce near 405 nm.[10,11] The observed spectral changes are suggestive of an alteration of

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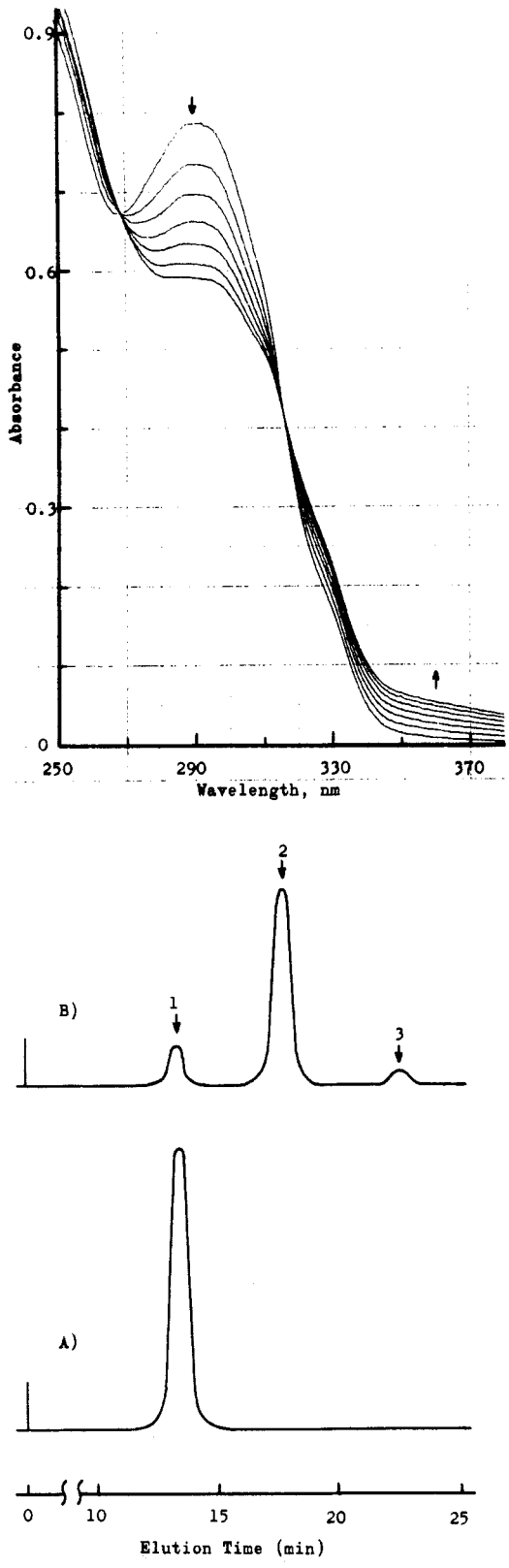


Figure 2. Change of electronic absorption spectrum of Cu(II)–PEM by photoirradiation at pH 7.6 (top) and HPLC analyses of UV-irradiated Cu(II)–PEM (B) and unirradiated Cu(II)–PEM (A). The time of photoirradiation was as follows: 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 h. The samples were placed on a Nova-PAK C_{18} column and eluted with the solvent (1% aqueous CH_3COONH_4–CH_3CN 84:16) at a flow rate of 2.0 mL/min. The eluate was monitored at 254 nm.

the bithiazole moiety of PEM by the light irradiation.

Figure 3 shows the 400-MHz ^1H NMR spectra of PEM and PEMH in the aromatic region (7.0–8.5 ppm). Each of the spectra exhibited four singlet signals (4 H) and multiplet phenyl reso-

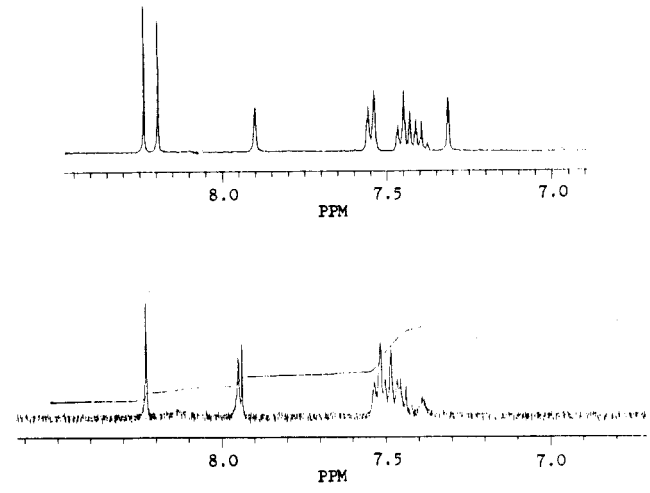


Figure 3. Spectra (400-MHz ^1H NMR) of the aromatic region of original (upper) and phototransformed (lower) PEMs in CD_3OD.

nances (5 H) in this region. In the spectrum of PEMH, the chemical shifts of two of the singlet signals, δ 7.39 and 7.95, were considerably shifted by changing the solvent from methanol-d_4 to D_2O, indicating that the two peaks in methanol-d_4 are assigned to the imidazole C5–H and C2–H protons, respectively. These chemical shift values are very close to those of the imidazole protons (δ 7.32 and 7.90) of PEM in methanol-d_4. Thus, the remaining two singlet signals (δ 7.94 and 8.23) must belong to the aromatic protons of the phototransformed bithiazole moiety of PEMH. PEM exhibits the two thiazole protons at δ 8.24 (C5–H) and 8.19 (C5′–H). Except for these aromatic protons in this region, all other protons of PEMH corresponded well to the respective protons of PEM. A similar ^1H NMR spectral change was observed in bleomycin A_2 (C5–H at δ 8.26 and C5′–H at δ 8.17 in methanol-d_4), which differs from PEM only in the terminal amine moiety and its photoproduct (two singlets at δ 8.22 and 7.95). The ^1H NMR spectra strongly indicate the occurrence of selective phototransformation of the bithiazole ring system. In the proton-decoupled ^{13}C NMR spectra, both PEM and PEMH gave the same number of carbon signals in methanol-d_4. Six carbons of the bithiazole rings of PEM are assigned to C2′ (δ 168.3, s), C4′ (δ 151.0, s), C5′ (δ 119.0, d), C2 (δ 164.1, s), C4 (δ 149.1, s), and C5 (δ 128.8, d) on the basis of multiplicities and chemical shifts compared with those of the model compounds.[12] On the other hand, PEMH exhibits six distinct aromatic carbons at δ 168.6 (s), 151.1 (s), 117.8 (d), 159.6 (s), 152.6 (s), and 122.1 (d). All of these carbons are assignable to the respective heteroaromatic carbons of the phototransformed bithiazole moiety of PEMH. Full assignment of these carbons in comparison with the data for the model compound will be discussed below. The ^1H and ^{13}C NMR data, coupled with the UV and fluorescence spectroscopic characteristics, evidently show that the photoirradiation of PEM results in a selective transformation of the bithiazole moiety without alteration of the other parts. In support of this, the 1:1 PEMH–Cu(II) complex revealed an ESR spectrum ($g_\parallel$ = 2.211, $g_\perp$ = 2.055, and $A_\parallel$ = 183 G) at pH 7.5 and 77 K which is identical with that of the parent PEM–Cu(II) complex,[13] indicating that the β-aminoalanine–pyrimidine–β-hydroxyhistidine portion as the metal chelation site suffers no significant alteration by the light irradiation.

In order to know the nature of the phototransformation of the bithiazole moiety in more detail, we investigated the photoreaction of BT (**3**), an adequate model for the bithiazole moiety of bleomycin antibiotics. Upon light irradiation in acetonitrile, this model compound exhibited a quite similar UV spectral change to that

(12) The previous ^{13}C signal assignment of the bleomycin bithiazole group was corrected from the present two-dimensional $^{13}C/^1H$ NMR data: Naganawa, H. In *Bleomycin: Chemical, Biochemical, and Biological Aspects*; Hecht, S. M., Ed.; Springer: New York, 1979; pp 106–123.

(13) Sugiura, Y.; Ishizu, K.; Miyoshi, K. *J. Antibiot.* **1979**, *32*, 453–461.

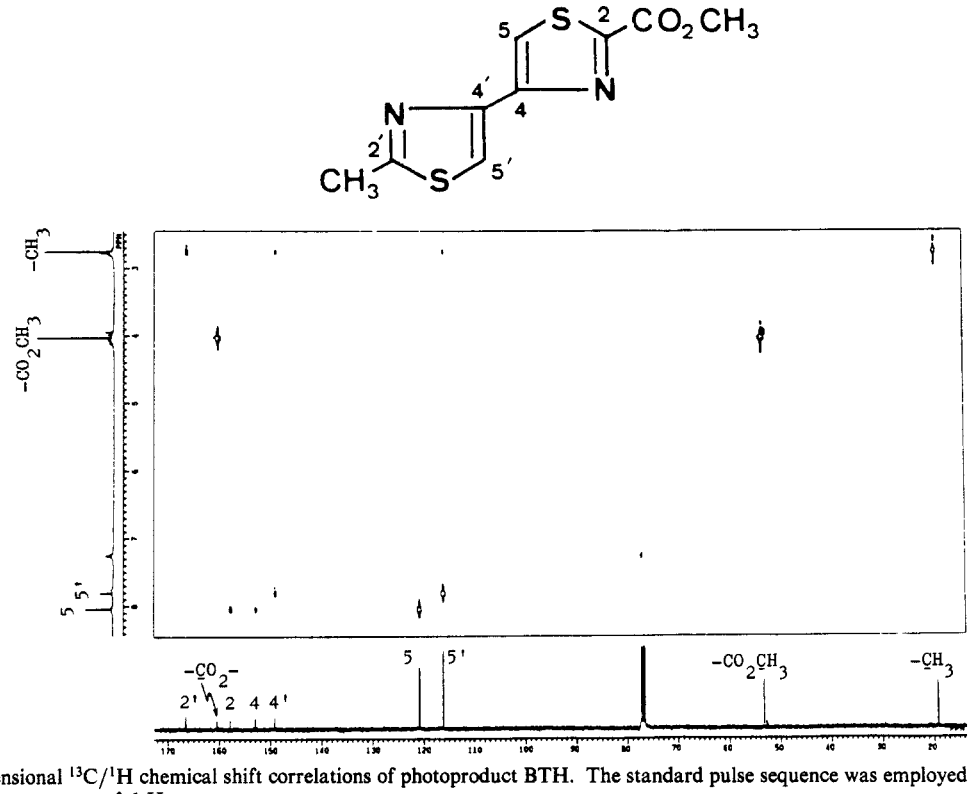
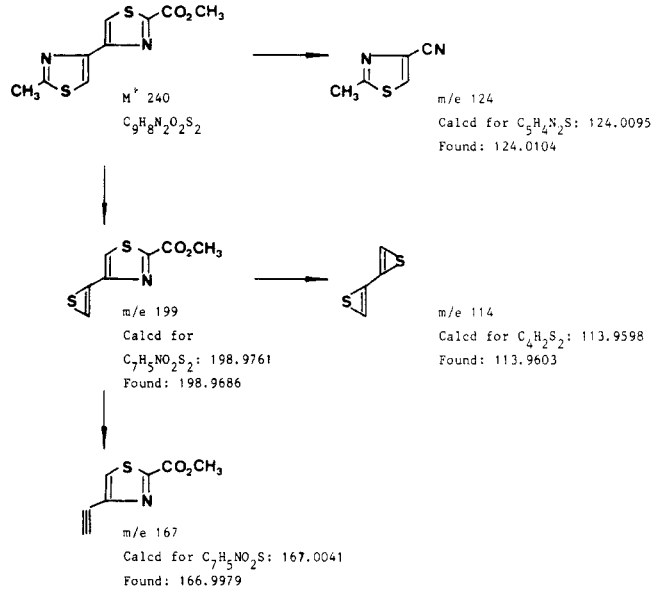


Figure 4. Two-dimensional $^{13}C/^{1}H$ chemical shift correlations of photoproduct BTH. The standard pulse sequence was employed with delays optimized for $^{13}C/^{1}H$ coupling constants of 6 Hz.

observed in the UV irradiation of PEM. HPLC analysis of the irradiation mixture also showed the presence of one major photoproduct which was easily isolated in a pure form by preparative HPLC. The analysis of the isolated photoproduct BTH (**4**) was as follows: mp 114–118 °C; UV (methanol) λ_{max} 293 nm (log ϵ 3.41), 248 (log ϵ 4.08); exact mass calcd for $C_9H_8N_2O_2S_2$ 240.0028, found 240.0053; MS (70 eV), *m/e* (relative intensity) 240 (M^+, 100), 209 (8), 199 (22), 182 (18), 167 (13), 155 (4), 141 (6), 140 (5), 124 (11), 114 (13), 96 (6); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.76 (s, 3 H), 4.03 (s, 3 H), 7.81 (s, 1 H), 8.06 (s, 1 H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 19.18 (q), 53.26 (q), 116.16 (d), 120.81 (d), 149.16 (s), 152.94 (s), 157.87 (s), 160.41 (s), 166.49 (s). The molecular formula ($C_9H_8N_2O_2S_2$) of the photoproduct (BTH) determined by high-resolution mass spectroscopy demonstrated that BTH is an isomer of BT. The 400-MHz ^1H NMR data of BTH were compared with those of BT. In analogy with the case of PEM, the bithiazole protons (C5–H at δ 8.17 and C5′–H at δ 7.98) of BT were shifted to a higher field in the spectrum (δ 8.06 and 7.81) of BTH in chloroform-d_1. The 100-MHz ^{13}C NMR spectroscopic studies including chemical shift comparison and multiplicities for both BT and BTH provided useful information for assigning the structure of BTH. BTH was assumed to possess two aromatic heterocycles as judged from its molecular formula and ^1H NMR data. In order to assign all the carbons rigorously, we employed the two-dimensional $^{13}C/^{1}H$ heteronuclear chemical shift correlation experiments using both large ($^1J_{C,H}$) and small (long-range, $^nJ_{C,H}$) C/H couplings. As shown in Figure 4, the $^{13}C/^{1}H$ 2D shift correlation map of BTH clarified that (1) the protons at δ 7.81 and 8.06 are linked to the carbons at δ 116.2 and 120.8, respectively, (2) the methyl protons at δ 2.76 have cross peaks with the carbon signals at δ 166.5, 149.2, and 116.2, and (3) the aromatic proton peak at δ 7.81 crosses with the carbon at δ 149.2. Of particular importance is the fact that three carbons at δ 166.5, 149.2, and 116.2 are on a methyl-substituted heteroaromatic ring, probably a thiazole ring. In BT (**3**), a similar long-range $^{13}C/^{1}H$ coupling pattern was observed between the methyl protons and the methyl-substituted thiazole ring carbons. In addition, the chemical shifts for three ring and methyl carbons of the photoproduct BTH are close to the respective carbons (δ 166.8, 148.7, 117.1, and 18.9) of BT. The results strongly indicate that BTH has a 2′-methyl 4′-substituted thiazole

Scheme I



structure. The methyl proton signal (δ 4.03) of the ester group gave a cross peak with the carbonyl carbon at δ 160.4 through a long-range $^3J_{C,H}$ coupling. Hence the remaining three carbons at δ 157.9 (s), 152.9 (s), and 120.8 (d) must belong to another heteroaromatic ring bearing a carbomethoxy group.

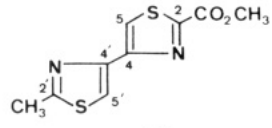
Further information on the structure of BTH was obtained by mass spectral fragmentations. The high-resolution mass spectrum of BTH showed the fragment ions at *m/e* 199, 167, 124, and 114 which are in accordance with the molecular formulas $C_7H_5NO_2S_2$, $C_7H_5NO_2S$, $C_5H_4N_2S$, and $C_4H_2S_2$, respectively. The fragment ions at *m/e* 199 and 167 strongly suggest the presence of a 2′-methyl 4′-substituted thiazole skeleton in BTH, as judged from the known mass spectral fragmentation pattern for thiazole derivatives,[14] consistent with the result from the $^{13}C/^{1}H$ 2D NMR

(14) Clark, G. M.; Grigg, R.; Williams, D. H. *J. Chem. Soc. B* **1966**, 339–343.

Table I. Representative 100-MHz ^{13}C NMR Spectral Data for BT (**3**), BTH (**4**), PEM (**1**), and PEMH (**5**)

	δ,[b] multiplicity[c]			
C[a]	BT (**3**)	BTH (**4**)	PEM (**1**)	PEMH (**5**)
2	163.4, s	157.9, s	164.1, s	159.6, s
4	147.2, s	152.9, s	149.1, s	152.6, s
5	128.7, d	120.8, d	128.8, d	122.1, d
2′	166.7, s	166.5, s	168.3, s	168.6, s
4′	147.8, s	149.2, s	151.0, s	151.1, s
5′	117.1, d	116.2, d	119.0, d	117.8, d

[a] Numbering is given in formulas **3** and **4**. [b] In chloroform-d_1. [c] Multiplicities were determined by proton-coupled spectrum. Abbreviations: s, singlet; d, doublet. [d] In methanol-d_4.



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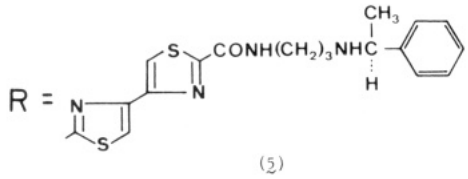

(5)

Figure 5. Proposed structures of photoproducts BTH (**4**) and PEMH (**5**).

spectroscopy. The appearance of the fragment ions at m/e 124 ($C_5H_4N_2S$) and 114 ($C_4H_2S_2$) is also indicative of the 4,4′-bithiazole structure for BTH (see Scheme I). Indeed, the mass spectrum of BT exhibited the fragmentation ions at m/e 199 and 167 but not at m/e 124 and 114.[15] Other structures such as 5,4′-bithiazole and carbomethoxyisothiazole cannot explain the appearance of both of the fragment ions at m/e 124 and 114. The structure possessing 1,2-location between the carbomethoxy group and aromatic proton was also eliminated by nuclear Overhauser effect (NOE) experiments of 2-(hydroxymethyl)-2′-methyl-4,4′-bithiazole[16] which was prepared by the treatment of BTH with excess $LiAlH_4$. No NOE was detected between the hydroxymethyl protons and the aromatic protons. All of the available evidence including spectroscopic data (UV, mass, and ^1H and ^{13}C NMR) are fully consistent with the proposed 4,4′-bithiazole structure (**4**) for the photoproduct BTH. Therefore, the phototransformation occurring in the light irradiation of the bleomycin model compound (**3**) has proven to be the photoisomerization of 2,4′-bithiazole to 4,4′-bithiazole. Such photorearrangement is indeed precedented for simple substituted thiazole derivatives.[17,18] For example, UV irradiation of 2-phenyl-4-methylthiazole is known to produce 3-phenyl-5-methylisothiazole as a major product and 4-phenyl-2-methylthiazole as a minor product.[17] Although the photoisomerization of BT (**3**) to BTH (**4**) is formally analogous to this type of known photorearrangement, it is of interest that the variation of major photoproduct was observed in the present case.

As already mentioned, the UV spectral change in the course of the photoirradiation of PEM (**1**) was remarkably similar to that observed in the UV irradiation of the model compound **3**. The major photoproduct of PEM, namely, PEMH, shows spectroscopic data, in particular ^1H and ^{13}C NMR data, comparable to those of BTH (**4**) (see Table I). These facts indicate that an analogous photorearrangement occurs at the bithiazole moiety of PEM. Since the structure of BTH was established as **4**, we tentatively assign the structure of PEMH as **5**, which possesses a 4,4′-bithiazole moiety as the DNA interaction site instead of the 2,4′-bithiazole structure of PEM (**1**) (see Figure 5). All the available data are consistent with this structure.

Herein, it is of special interest to examine the DNA cleaving reaction of PEMH, because it contains a modified DNA binding

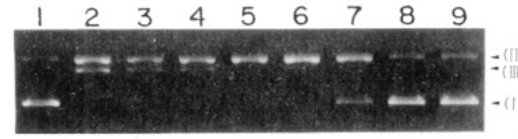

Figure 6. Agarose gel electrophoretic patterns of ethidium bromide stained ϕ×174 RF DNA after treatment with iron and cobalt complexes of PEM, PEMH, and phleomycin. The samples contained 0.3 μg of DNA, 20 mM Tris–HCl buffer (pH 7.5), and the following additions: lane 1, none; lane 2, 0.5 μM Fe(II)–PEM plus 50 μM 2-mercaptoethanol; lane 3, 0.5 μM Fe(II)–PEMH plus 50 μM 2-mercaptoethanol; lane 4, 0.5 μM Fe(II)–phleomycin plus 50 μM 2-mercaptoethanol; lane 5, 1 μM Co(III)–PEM; lane 6, 1 μM Co(III)–PEMH; lane 7, 1 μM Co(III)–phleomycin; lane 8, 0.5 μM Fe(II) plus 50 μM 2-mercaptoethanol; and lane 9, 1 μM Co(III). The reaction samples were incubated at 20 °C for 8 min (lanes 2–4 and 8) or irradiated at a distance of 6 cm from the transilluminator at 0 °C for 6 min (lanes 5–7 and 9).

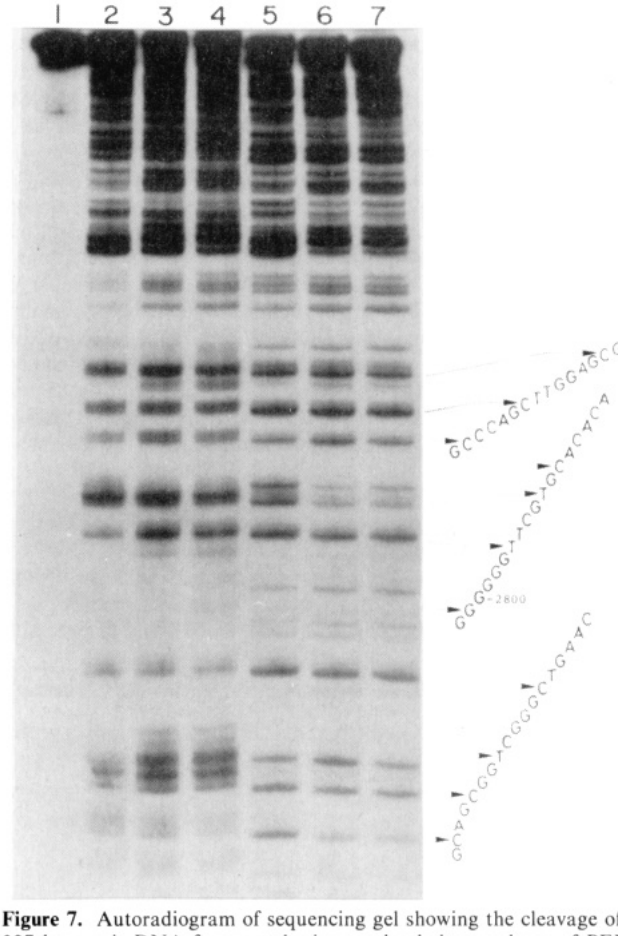

Figure 7. Autoradiogram of sequencing gel showing the cleavage of a 327 base pair DNA fragment by iron and cobalt complexes of PEM, PEMH, and phleomycin. The reaction samples contained a 5′-end ^{32}P-labeled DNA fragment, 1 μg of sonicated calf thymus DNA, 20 mM Tris–HCl buffer (pH 7.5), and the following additions: lane 1, none; lane 2, 10 μM Co(III)–phleomycin; lane 3, 10 μM Co(III)–PEMH; lane 4, 10 μM Co(III)–PEM; lane 5, 5 μM Fe(II)–phleomycin plus 1 mM 2-mercaptoethanol; lane 6, 5 μM Fe(II)–PEMH plus 1 mM 2-mercaptoethanol; and lane 7, 5 μM Fe(II)–PEM plus 1 mM 2-mercaptoethanol. The sequence is indicated on the right of the figure, and the number corresponds to the nucleotide number in the pBR322 entire sequence map.[22]

(15) BT (**3**): MS (70 eV), m/e (relative intensity) 240 (M^+, 100), 209 (28), 199 (25), 182 (67), 167 (14), 141 (14), 125 (3), 116 (2).

(16) Light-yellow powder; mp 150–153 °C; exact mass calcd for $C_8H_8N_2OS_2$ 212.0064, found 212.0065; MS (70 eV), m/e (relative intensity) 212 (M^+, 100), 183 (38), 171 (8), 155 (4), 153 (6), 142 (11), 141 (9), 125 (2), 124 (2), 114 (13); ^1H NMR ($CDCl_3$) δ 2.75 (s, 3 H), 4.80 (br s, 1 H), 4.98 (s, 2 H), 7.58 (s, 1 H), 7.72 (s, 1 H).

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site. Figure 6 compares the DNA cleavage activities by iron and cobalt complex systems of PEM, PEMH, and phleomycin by using an agarose gel electrophoretic experiment. In both of the metal complex systems, PEMH clearly showed activity similar to that of PEM. The DNA breakage activity by the phleomycin–Co(III) UV system was somewhat lower than those by the corresponding PEM and PEMH systems. In the DNA strand scission, the nucleotide sequence specificity by the Fe(II)–oxygen reductant and the Co(III) UV light systems of PEMH was determined and compared with those induced by the corresponding PEM and phleomycin complexes (Figure 7). The PEMH–metal complex systems preferentially attacked G-C (5′ → 3′) and G-T (5′ → 3′) sequences. Of special interest is the fact that these compounds revealed almost identical cleavage patterns in the DNA fragment (327 base pairs) labeled at the 5′ terminus. The phototransformed bleomycin A_2 also showed the guanine–pyrimidine (5′ → 3′) sequence specificity to be the same as that of the parent bleomycin A_2. In addition, these observations were confirmed by the experiments using a 301 base pair DNA fragment. The present results demonstrate that the alteration of the 2,4′-bithiazole ring system to 4,4′-bithiazole has no effect on the nucleotide sequence specificity in the DNA cleaving reaction of bleomycin antibiotics. The space-filling model revealed that the planarity of the 4,4′-bithiazole ring system is almost the same as that for the 2,4′-bithiazole ring system. Indeed, X-ray crystallographic analysis of the bleomycin bithiazole model compound, 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylic acid, clarified that the bithiazole ring system is planar.[19] The bithiazole moiety of the bleomycin molecule is known to contribute significantly to DNA interaction. A recent study on the interaction of some 2′,4-disubstituted bithiazoles with DNA suggested that (1) efficient DNA binding requires at least two aromatic rings, since no binding to poly(dA-dT) is observed for monothiazole model compounds, and (2) even phenyl–thiazole derivatives containing two different aromatic rings undergo intercalation into DNA.[20] The basic requirement for a DNA intercalater is that it be planar and aromatic. In PEM, PEMH, and phleomycin, the site specificity of DNA strand scission was unchanged. The phototransformed PEM and phleomycin evidently differ from the original PEM in that the 2,4′-bithiazole ring system is replaced by 4,4′-bithiazole and 2-thiazolinylthiazole heterocycles. Such a structural change gives no significant effect on the nucleotide sequence specificity of DNA degradation. The present observations suggest that (1) the bithiazole moieties of 2,4′ and 4,4′ linkages have almost the same DNA binding affinity[21] and cleavage specificity and (2) intercalation of the conjugated 2,4′-bithiazole group may be not a critical factor in directing the site specificity of DNA strand scission by bleomycin antibiotics. It should be also noted that the phleomycin–Co(III) UV light system clearly reduced the DNA cleavage activity in comparison with the corresponding PEM and PEMH systems. In the Co(III) UV light system of bleomycin antibiotics, the presence of bithiazole rings appears to be required for efficient DNA cleavage.

The 1:1 bleomycin–Co(III) complex has DNA cleaving activity that is induced only by UV irradiation.[5] The photoirradiation of the PEM–Co(III) complex also gave one major HPLC peak. Together with structural analysis of a minor photoproduct, the X-ray crystallographic study of the major photoproduct is currently under way.

In summary, the present study demonstrated the following conclusions: (1) the bithiazole moiety is the photoreactive site in bleomycin antibiotics, (2) the thiazole ring linked to the terminal side chain undergoes photoisomerization, and (3) the phototransformed bleomycin has a nucleotide sequence cleavage mode almost identical with that of the original bleomycin. These results give valuable information not only for photoreactivity of bleomycin antibiotics but also for nucleotide sequence recognition by the bithiazole group. Furthermore, the photochemistry of bleomycin provides a convenient and powerful tool for specific modification of the bithiazole part of other bleomycin derivatives of medicinal importance.

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Registry No. 3, 80337-72-0; **4**, 104267-23-4; **5**, 104267-24-5; bleomycin, 11056-06-7.

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(21) In 20 mM Tris (pH 7.5), the binding constant of PEM was estimated to be $k_{app} = 5.6 \times 10^4\ M^{-1}$ from the fluorescence quenching experiment using calf thymus DNA (0.43 mM). The preliminary result of competitive reaction for DNA showed that PEMH has a DNA binding affinity very close to that of PEM. By equilibrium dialysis method, the determination of the accurate binding constant is currently under way.

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