Synthesis and Characterization of Ethidium Analogs: Emphasis on Amino and Azido Substituents [1]

William J. Firth, III [2], Charles L. Watkins [3], David E. Graves [4], and Lerena W. Yielding [2,5]

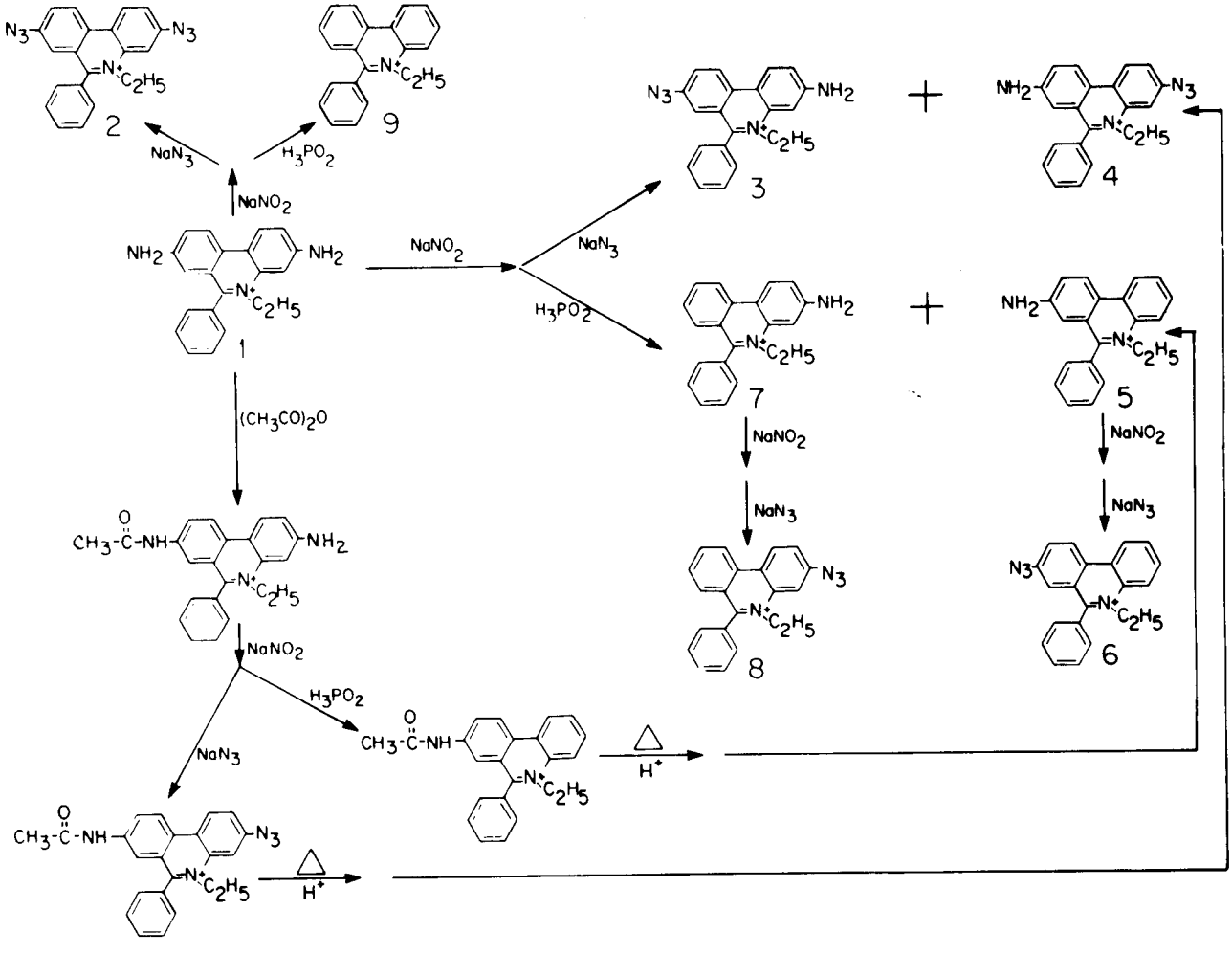
Department of Biochemistry, University of South Alabama, Mobile, AL 36688

Received November 8, 1982

A series of eight ethidium derivatives has been synthesized in which the substituents at R3 and R8 have been varied with hydrogen, azido and amino functions. Three of these compounds are new and their synthesis and characterization by uv-visible, ir and ^{1}H nmr spectroscopy are presented. The synthesis and characterization of the other compounds are also given for comparison, because the compounds served as precursors, or the synthetic route undertaken for these compounds differed from that reported previously.

J. Heterocyclic Chem., **20**, 759 (1983).

Ethidium bromide (**1**) has been used extensively as a trypanocidal agent. In addition, its interaction with DNA has been extensively investigated and has contributed to its value as a biological probe for characterizing nucleic acid structure and function. Unfortunately, the reversible nature of these interactions precludes the isolation of the targets responsible for its biological actions. Photoaffinity labeling, involving the design and synthesis of analogs, which can covalently attach at their biological binding sites when photoactivated to a reactive form, provides a means of identifying those sites. In the course of developing photoaffinity probes for the biological actions of ethidium bromide, the substituent requirements for the biological activity of ethidium had to be determined. A series of ethidium analogs emphasizing amino, azido and hydrogen substitutions at R_3 and R_8 were prepared. The structure-function relationships were examined, and the results emphasized the importance of 1) the amino substituents for



biological activity and 2) the azido substituent as an effective means of covalently attaching the ligand for enhanced biological potential [6-8]. Furthermore, at least two photoaffinity probes for ethidium have been identified [9-11]. In this presentation, the synthesis of these analogs is described. The ^1H nmr spectroscopy has confirmed the structures of all the compounds, and has demonstrated consequently that isomers were obtained when ethidium (**1**) was diazotized with one equivalent of sodium nitrite, whereas, monoacetylation of ethidium provided selectively the 8-acetamido derivative.

The uv-visible transitions of ethidium and its various amino, azido and hydrogen substituted analogs have provided a means of compound identification. Table I shows the effects of substitution at the R_3 and R_8 phenanthridinium ring positions on the uv-visible absorption maxima. The parent phenanthridinium compound **9** with hydrogen substituents at R_3 and R_8 exhibited a strong absorption band at 321 nm and a doublet peak at 363 nm and 377 nm. Addition of a single amino substituent at either R_3 or R_8 shifted the visible absorption maximum bathochromically to 437 nm for the 3-amino analog **7** and to 430 nm for the 8-amino-analog **5**, respectively. In addition, the 3-amino-analog **7** demonstrated a uv transition at 242 nm which was not observed for the 8-amino-analog **5**. These results [except for the molar absorptivity of the 3-amino-analog **7**] are in agreement with those of Zimmermann and Zimmermann [12]. The ethidium bromide [1] demonstrated an additional bathochromic shift to 478 nm because of amino substitutions at both R_3 and R_8.

The heterosubstituted analogs, *i.e.*, 3-azido-8-amino-**4** and 8-azido-3-amino-**3** isomers showed similar visible absorption spectra with absorption maxima at 454 nm and 462 nm. The desamino-azido isomers, 3-azido-**8** and 8-azido-5-ethyl-6-phenylphenanthridinium chloride **6**, also exhibited similar visible spectra with the absorption maxima shifted bathochromically from the parent phenanthridinium compound **9** to 400 nm and 406 nm, respectively. These analogs could be distinguished by uv-transitions occurring at 245 nm for the 3-azido-analog **8** and at 207 nm for the 8-azido-analog **6**. Addition of azido substituents at both R_3 and R_8 resulted in a bathochromic shift of the major visible transition to 432 nm when compared to the deaminated analog **9**.

The fingerprint region of the uv spectra of these phenanthridinium compounds was characterized by a 284-288 nm maximum usually 9-10 times greater than the visible absorption maxima if there were amino and/or azido substituents at R_3 and R_8 (Table 1). The uv spectra of phenanthridinium compounds with either an amino or an azido substituent at R_3 and R_8 were characterized by a maximum at 270-275 nm; *i.e.*, the 8-amino-**5** and 8-azido-**6** analogs had a 270 nm maximum, the 3-amino-**7** and 3-azido-**8** analogs showed a maximum at 275 nm and 274 nm, respectively. The uv spectrum reflected an absorption peak at 242-251 nm if there was not an amino or azido

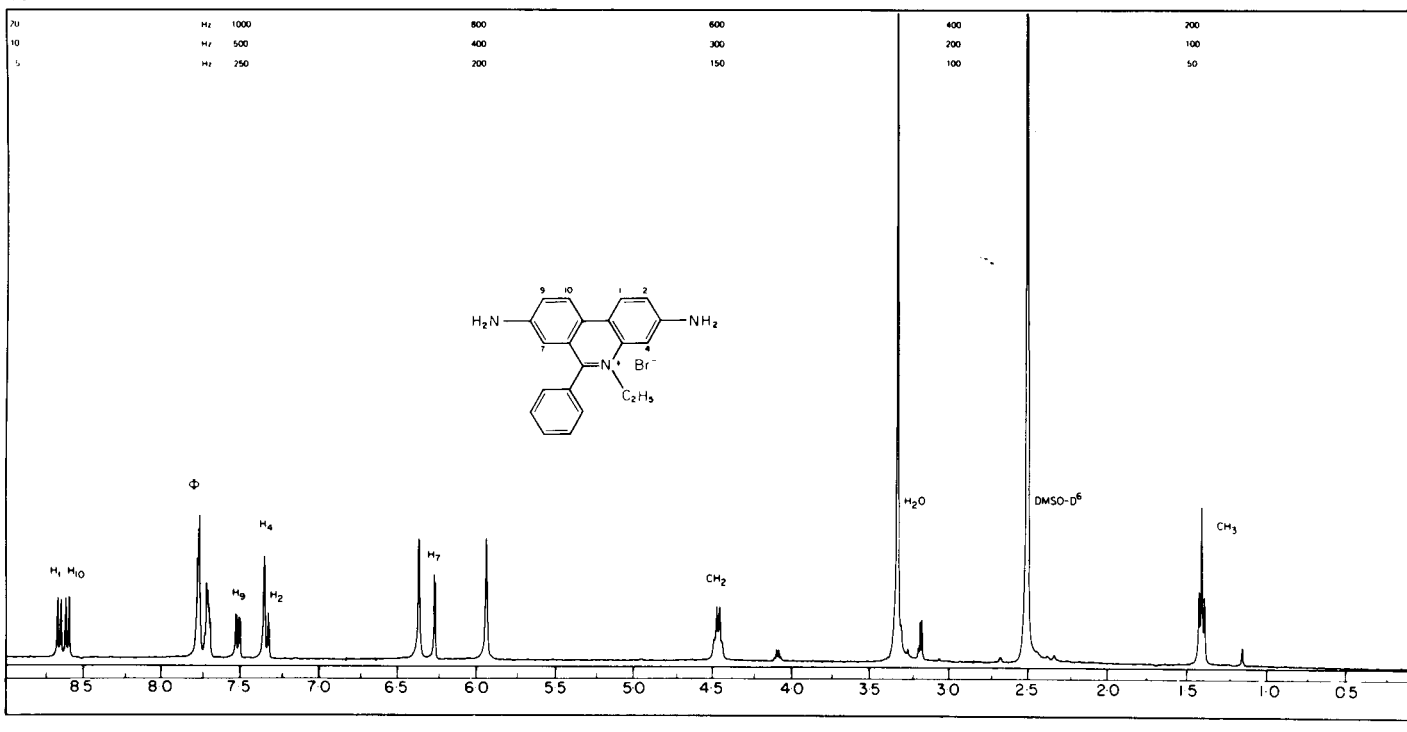


Figure 1. The 400 MHz proton Fourier transorm nmr spectrum of ethidium bromide (5 mM) in DMSO-d_6 at 25°; TMS used as the internal reference.

Table I

UV-Visible Spectrophotometric Characterization of Ethidium Analogs

Compound	Visible (nm) λ max	Visible ($M^{-1}cm^{-1}$)	Ultraviolet (nm) λ max	Ultraviolet ($M^{-1}cm^{-1}$)	Photolytic Rate Constant (sec^{-1})	Half-life (sec)
1	478	5680	285/213	56,800/44,300	—	—
2	432	5850	294/284	60,210/52,480	1.14×10^{-1}	6.08
3	462	5220	288/214	53,770/36,540	2.56×10^{-3}	2.71×10^{2}
4	454	5190	287/214	55,530/35,810	1.71×10^{-2}	4.05×10^{1}
5	430	3380	270	39,190	—	—
6	406	4250	270/207	45,900/35,700	7.33×10^{-3}	9.45×10^{1}
7	437	4500	275/242	32,860/23,990	—	—
8	400	6410	270/245	50,000/25,000	1.1×10^{-1}	6.33
9	377/363	4770/4930	321/251	7,700/43,040	—	—

The UV-visible spectrophotometric analyses of the ethidium analogs was performed on a Cary 219 recording spectrophotomer. Darkroom conditions were maintained when the azido analogs were being examined. All compounds were dissolved in *p*H 3 water (HCl) and photoactivated using 2 GE daylight fluorescent lightboxes delivering 80 J/(m^2sec), at 0°. Molar absorptivities are based on the molecular weights determined from elemental analysis for the nonazido analogs. The molecular weights of the azido compounds were approximated by assuming 1 mole of water which was found for the non-azido compounds.

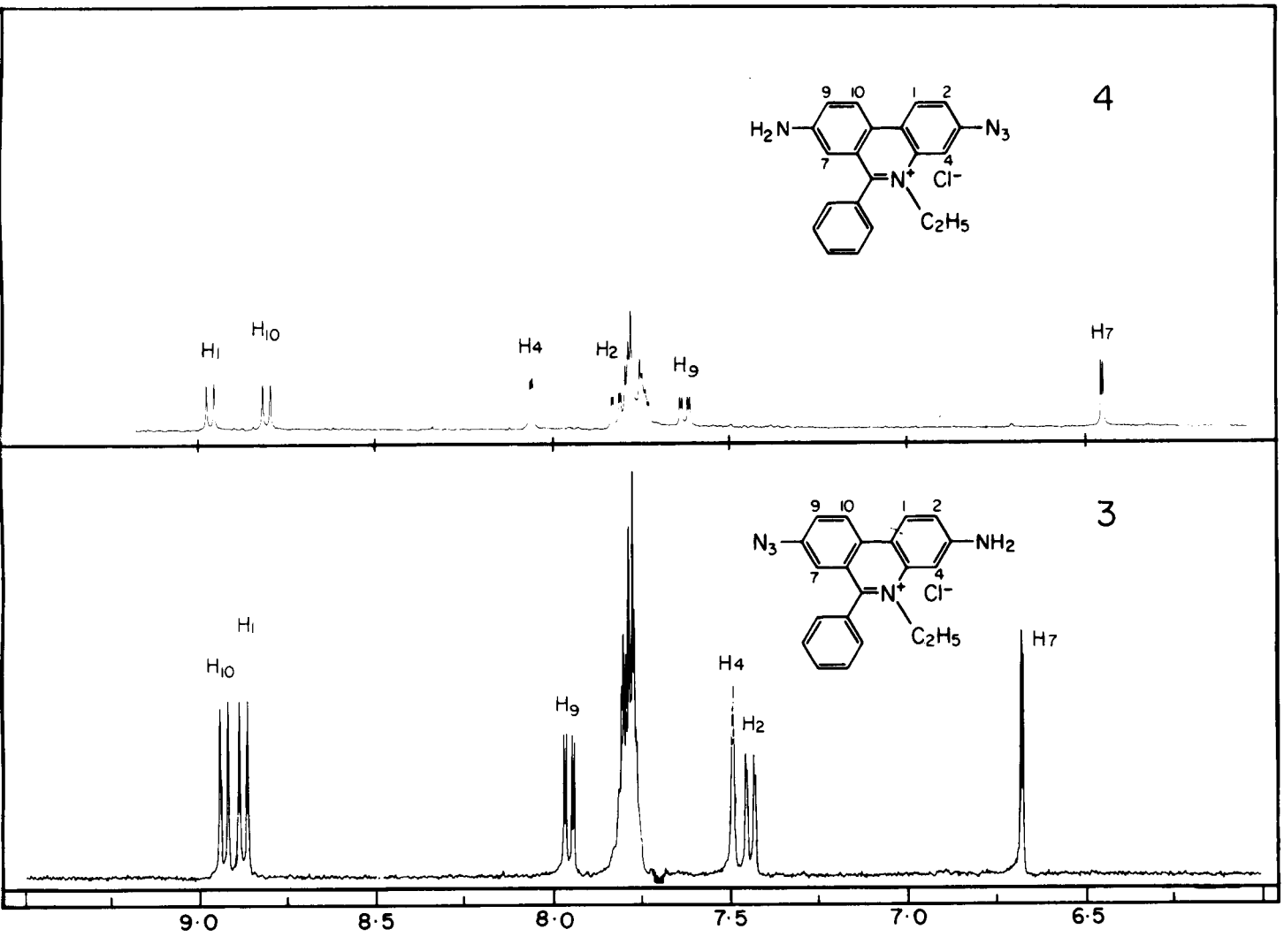


Figure 2. The 400 MHz proton nmr spectra of the aromatic regions of the hetero-substituted 3-azido-8-amino-5-ethyl-6-phenylphenanthridium chloride (**4**) and 3-amino-8-azido-5-ethyl-6-phenylphenanthridinium chloride, (**3**) in DMSO-d_6.

substituent at the R_8; *i.e.*, the 3-amino-**7** and 3-azido-**8** analogs demonstrated 242 nm and 245 nm peaks, respectively. The parent phenanthridinium compound **9**, which had only hydrogen substituents at both R_3 and R_8, had a 251 nm peak.

Generally, photoactivation of the azido analogs in water yielded compounds which could not be activated further by light. These inactive compounds demonstrated visible transitions in the absorption spectra which were both bathochromic and hyperchromic compared to those of their active precursors. The uv region of the absorption spectra of the azido analogs and their subsequent photoinactivated species were very similar and will not be further described. The rates of photoactivation are given in Table I and comparison reveals that photoactivation was more rapid for an azido group at R_3 than at R_8, which is probably due to the position of electrophilic azido moiety with respect to the positively charged quaternary nitrogen. This nitrogen directs resonance delocalization and subsequent nitrene activation.

The ir analysis of the ethidium analogs was useful for structure confirmation (Table II). Characteristic N—H stretching vibrations for primary amines were observed in the 3300-3200 cm^{-1} region. Strong absorption occurred at 1625 cm^{-1} due to the N—H bending and at 1250 cm^{-1} for C—N stretching vibrations, and these peaks were absent for the deaminated analog (9). The C—H bending modes of the monosubstituted phenyl group occur at 700 cm^{-1} and 750 cm^{-1}. Azido analogs displayed strong absorption at 2100 cm^{-1}. The fingerprint region was variable and highly characteristic of each analog.

The 400 MHz ^1H nmr spectrum of ethidium (**1**) (0.005*M*) in DMSO-d_6 is shown in Figure I and each of the phenanthridinium ring protons were assigned to a given resonance. The ^1H chemical shifts of the ring protons were found to be concentration dependent above 0.025*M*; therefore, all chemical shifts reported in Table III were extrapolated to infinite dilution. The coupling constants are given in Table IV. The chemical shift assignments for ethidium bromide (**1**) were based on the observation that the H_7 proton is situated in the shielding region of the phenyl group attached at C_6 of the phenanthridinium ring, the phenyl group being perpendicular to the ring [13-16]. The H_7 proton showed a small *meta* coupling to H_9, J_{HH} = 2.44 Hz (Table IV). Homonuclear spin-spin decoupling experiments, therefore, gave the assignments of H_9 and H_{10}. Likewise, the H_1, H_2 and H_4 assignments were made. The five protons from the monosubstituted phenyl group showed a complicated pattern characterized by two groups of resonances at 7.700 and 7.63 pppm. The chemical shift assignments were consistent with those previously reported [15,16] with the exception of a 100 MHz nmr study of ethidium in DMSO-d_6 solution (17) where the H_2 and H_9 resonances were reversed.

Table II

Compound	Rf	IR (cm^{-1}) (KBr)	Analysis Found	Analysis Calcd.	Formula
1	0.1	3300-3150	—	—	$C_{21}H_{20}ClN_3$
2	0.9	2130	—	—	$C_{21}H_{16}ClN_7 \cdot H_2O$
3	0.45	3400-3300 2130	—	—	$C_{21}H_{18}ClN_5 \cdot H_2O$
4	0.5	3200-3300 2080	—	—	$C_{21}H_{18}ClN_5 \cdot H_2O$
5	0.45	3400	71.31 C 7.68 N 5.99 H	71.48 C 7.94 N 6.00 H	$C_{21}H_{19}ClN_2 \cdot H_2O$
6	0.9	2000	—	—	$C_{21}H_{17}ClN_4 \cdot H_2O$
7	0.5	3300	71.38 C 7.71 N 5.98 H	71.48 C 7.94 N 6.00 H	$C_{21}H_{19}ClN_2 \cdot H_2O$
8	0.9	2130	—	—	$C_{21}H_{17}ClN_4 \cdot H_2O$
9	0.9	absence of 2130 absence of 3300	74.59 C 4.02 N 5.96 H	74.66 C 4.15 N 5.97 H	$C_{21}H_{18}ClN \cdot H_2O$

Deamination of the phenanthridinium ring at R_3 or R_8 caused downfield shifts for all the ring protons in the ^1H nmr spectrum, the changes at H_7 (R_8 = H, R_3 = NH_2 or H), and at H_4, (R_3 = H, R_8 = NH_2 or H), being greater than that expected on the basis of substituent effects alone, as observed for monosubstituted benzenes, *i.e.*, aniline *versus* benzene [18]. Interestingly, the magnitude of the downfield chemical shifts at H_7 and H_9 (substitution at R_8, H for NH_2) and at H_2 and H_4 (substitution at R_3, H for NH_2) could be directly correlated with π-electron density changes at the associated carbon atoms using the S C F —
CI—PPP calculations of Zimmermann and Zimmerman [12]. The complex resonance pattern demonstrated for the phenyl protons when R_8 = N_3 or NH_2 collapsed to a single sharp resonance when R_8 = H. The aromatic region of the ^1H nmr spectrum for compound **7** (R_3 = NH_2, R_8 = H), was first order, and could be easily assigned by homonuclear spin-spin decoupling and substituent effect considerations. The aromatic region of compounds **5** (R_3 = H, R_8 = NH_2) and **9** (R_3 = R_8 = H) were complicated by the fact that H_1, H_2, H_3 and H_4 formed a non-first order pattern, ABMX, with H_2 and H_3 constituting th AB part. This pattern could be reduced to two ABX type patterns by decoupling at H_1 or H_4. The spectral parameters were then calculated using standard techniques [19]. Substitution of the azido function, N_3, at R_3 or R_8 for NH_2 caused downfield shifts for all the ring protons, those *ortho* and *meta* to the azido function experiencing the greatest change. The assignments given in Table III and shown in Figure 2 of the aromatic region only of the two isomers **3** (R_8 = N_3, R_3 = NH_2), and **4** (R_8 = NH_2, R_3 = N_3), are based on spin decoupling experiments, the crystal structure geometry of

Table III

^{1}H NMR Chemical Shifts for Ethidium Bromide and Eight Derivatives in DMSO-d_6 Solution

R	Compound								
	1	**2**	**3**	**4**	**5**	**6**	**7** (a)	**8** (b)	**9**
CH_3	1.402 (t)	1.448 (t)	1.446 (t)	1.400 (t)	1.431 (t)	1.485 (t)	1.446 (t)	1.450 (t)	1.484 (t)
CH_2	4.458 (q)	4.818 (q)	4.540 (q)	4.697 (q)	4.716 (q)	4.841 (q)	4.532 (q)	4.812 (q)	4.828 (q)
H_7	6.261 (d)	6.831 (d)	6.680 (d)	6.418 (d)	6.452 (d)	6.869 (d)	7.288 (dd)	7.488 (dd)	7.524 (dd)
H_8	—	—	—	—	—	—	7.615 (ddd)	7.891 (ddd)	7.934 (ddd)
H_9	7.511 (dd)	8.158 (dd)	7.955 (dd)	7.620 (dd)	7.635 (dd)	8.178 (dd)	8.126 (ddd)	8.345 (ddd)	8.367 (ddd)
H_{10}	8.604 (d)	9.230 (d)	8.932 (d)	8.848 (d)	8.889 (d)	9.297 (d)	8.878 (d)	9.197 (dd)	9.250 (dd)
H_1	8.657 (d)	9.279 (d)	8.877 (d)	9.013 (d)	9.004 (dd)	9.286 (dd)	8.899 (d)	9.316 (d)	9.309 (dd)
H_2	7.330 (dd)	7.980 (dd)	7.441 (dd)	7.830 (dd)	8.004 (m)	8.171 (m)	7.434 (dd)	7.960 (dd)	8.176 (m)
H_3	—	—	—	—	7.953 (m)	8.186 (m)	—	—	8.206 (m)
H_4	7.341 (d)	8.236 (d)	7.483 (d)	8.077 (d)	8.545 (dd)	8.728 (dd)	7.508 (d)	8.239 (d)	8.725 (dd)
Phenyl	7.700 (m)	7.816 (m)	7.775 (m)	7.759 (m)	7.790 (m)	7.826 (m)	7.777 (m)	7.819 (m)	7.823 (s)
	7.763 (m)		7.800 (m)						

The chemical shifts are reported in ppm, from TMS, and are the values extrapolated to infinite dilution.
(a) Compound **7** was found 95% pure with the major contaminant being Compound **5**. (b) Compound **8** was found 95% pure with the major contaminant being Compound **6**.

Table IV

Coupling Constants for Ethidium Bromide and Eight Compounds in DMSO-d_6 Solution Based on ^{1}H NMR Spectra

Compounds: **1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
J = 7.3 (CH_2CH_3)	J = 7.0	J = 7.0	J = 7.1	J = 7.1	J = 7.1	J = 7.1	J = 7.1	J = 7.3
$J_{7,9}$ = 2.4	$J_{7,9}$ = 2.4	$J_{7,9}$ = 2.3	$J_{7,9}$ = 2.3	$J_{7,9}$ = 2.3	$J_{7,9}$ = 2.4	$J_{7,9}$ = 1.0	$J_{7,9}$ = 1.0	$J_{7,9}$ = 1.0
$J_{9,10}$ = 9.1	$J_{9,10}$ = 9.0	$J_{9,10}$ = 9.15	$J_{9,10}$ = 9.15	$J_{9,10}$ = 8.9	$J_{9,10}$ = 8.8	$J_{9,10}$ = 7.8	$J_{9,10}$ = 8.4	$J_{9,10}$ = 8.3
$J_{1,2}$ = 8.8	$J_{1,2}$ = 9.0	$J_{1,2}$ = 9.3	$J_{1,2}$ = 9.1	$J_{1,2}$ = 8.3	$J_{1,2}$ = 7.6	$J_{1,2}$ = 9.2	$J_{1,2}$ = 8.9	$J_{1,2}$ = 8.1
$J_{2,4}$ = 1.5	$J_{2,4}$ = 2.1	$J_{2,4}$ = 2.0	$J_{2,4}$ = 2.0	$J_{2,4}$ = 0.7	$J_{2,4}$ = 2.7	$J_{2,4}$ = 1.7	$J_{2,4}$ = 2.05	$J_{2,4}$ = 0.7
				$J_{1,3}$ = 1.0	$J_{1,3}$ = 2.0	$J_{7,8}$ = 8.3	$J_{7,8}$ = 8.2	$J_{7,8}$ = 8.3
				$J_{2,3}$ = 7.1	$J_{2,3}$ = 7.0	$J_{8,9}$ = 6.8	$J_{8,9}$ = 7.1	$J_{8,9}$ = 7.3
				$J_{3,4}$ = 9.0	$J_{3,4}$ = 7.3	$J_{8,10}$ = 1.0	$J_{8,10}$ = 1.0	$J_{8,10}$ = 1.0
								$J_{1,3}$ = 1.3
								$J_{2,3}$ = 7.1
								$J_{3,4}$ = 8.7

the compound [20] and previous nmr studies [21]. The ^{1}H nmr spectral assignments for compounds **2** (R_3 = R_8 = N_3), **6** (R_8 = N_3, R_3 = H), and **8** (R_8 = H, R_3 = N_3), given in Table III were derived from substituent effect considerations (H for NH_2, N_3 for NH_2) in compounds **3, 4, 6, 7** and **9**. Compound **6** showed a non-first order ABMX spectrum for H_1-H_4. The same procedure previously employed for compounds **5** and **9** was followed for the calculation of the chemical shifts and coupling constants. Thus, all the ^{1}H chemical shifts and coupling constants were determined for each of the nine phenanthridinium compounds (Tables III and IV). In addition, the spectral positions were sufficiently distinct for each compound that identification and determination of purity of reaction mixtures, including differentiation of isomers (Figure 2), was easily effected using ^{1}H nmr.

In conclusion, we have shown that diazotization of ethidium yielded isomeric products, although the 8-diazonium analog was always the predominant product over the 3-diazonium isomer. The nmr spectroscopy has provided conclusive evidence for the presence of isomers when diazotization was not preceded by monoacetylation, which selectively protects the 8-amino substituent. The presence of the 3-acetamido derivative was not detected presumably because of the participation of the 3-amino group in a resonance configuration with the quaternary nitrogen, making it unavailable for attack by the acetylating agent.

EXPERIMENTAL

Due to the highly reactive nature of the azido analogs, elemental analysis was not possible. Molecular weights for these compounds was based on one molecule of water of hydration which was found from the elemental analysis of the non-azido compounds. The uv-visible absorption spectra were obtained using a Cary 219 recording spectrophotometer for 5 × $10^{-5}M$ aqueous solutions (*p*H 3.0) of all the analogs. From these data, molar absorptivities were determined for each analog using a Beer-Lambert plot (Table I). All handling of photosensitive compounds was performed under safelight. Photoactivation was achieved using two light boxes (Buchler Inst.), each equipped with two GE daylight #F15-T8D

lightbulbs. Energy was delivered at a rate of 80 J/(m²·sec). All samples ($5 \times 10^{-5}M$) were irradiated simultaneously and maintained at 0° throughout the irradiation. The observed spectral change *versus* photoactivation time was characterized by first-order kinetics yielding a rate constant of photoactivation for each analog [22]. Infrared spectroscopy was performed on an Acculab 4 (Beckman) grating instrument (Table II). Spectra were determined on 2 mm potassium bromide pellets (5 mg of sample per 100 mg of alkali halide) and were referenced to the 1028.3 cm^{-1} absorption peak of polystyrene. The ^1H nmr spectra of the nine compounds were obtained on a Bruker WH-400 superconducting nmr spectrometer (Comprehensive Cancer Center, University of Alabama in Birmingham Medical Center, Birmingham, Alabama) operating at 400.134 MHz in the Fourier Transform mode at 25° with dimethyl sulfoxide-d_6 (Merck) as solvent. The DMSO-d_6 also served as an internal deuterium lock source while tetramethylsilane was employed as the internal reference. The free induction decay consisted of 32K data points, yielding a real spectrum of 16K data points. The spectral width was 4000 Hz, giving a computer resolution of 0.244 Hz per data point. The precision of the chemical shifts may be taken to be ±0.001 δ. Sixty-four scans were accumulated for each spectrum. Since ethidium bromide has been observed to associate strongly in aqueous solution, the concentration dependence of the chemical shifts of all the compounds was measured, and all ^1H nmr chemical shifts in Table III are reported at infinite dilution. Typically, samples of 0.002 to 0.01*M* were used in the study. The ^1H nmr homonuclear spin-spin decoupling experiments were used extensively in the assignment of the individual phenanthridinium ring protons. The ^1H nmr coupling constants are given in Table IV.

General Procedure for Diazotization.

All synthesis procedures were carried out under photographic safelights. A solution of ethidium bromide in 0.1 *N* hydrochloric acid was cooled to 0° in an ice bath. Sodium nitrite was dissolved in 0.1 *N* hydrochloric acid was cooled to 0° in an ice bath. Sodium nitrite was dissolved in 0.1 *N* hydrochloric acid (0°) just prior to its dropwise addition to the continuously stirring solution of ethidium. The reaction proceeded in the dark for 3-10 minutes. In some cases sulfamic acid was added to destroy unreacted nitrous acid. When an azido derivative was desired, sodium azide was dissolved in 0.1 *N* hydrochloric acid (0°) and added dropwise to the diazotization reaction, which continued stirring overnight at 0° in the dark. When deamination was sought, hypophosphorous acid was included in the ethidium solution which was cooled at 0° prior to the sodium nitrite addition, and this reaction was allowed to continue in the dark at 0° for 1-2 days.

General Procedure for Purification.

Cation exchange chromatography with carboxymethylcellulose (Bio-rad-Cellex CM) was used for purification. Fractions (100 ml) were eluted with aqueous hydrochloric acid (*p*H 4.5-2.0) and analyzed spectrophotometrically (uv-visible). The fractions were lyophilized and subsequently checked for purity by thin layer chromatography using Silica Gel G (chloroform/ethanol/acetic acid, 4:1:1).

Ethidium Bromide: 3,8-Diamino-5-ethyl-6-phenylphenanthridinium Chloride (**1**).

Ethidium (Calbiochem) was recrystallized from methanol to give maroon needles.

3,8-Diazido-5-ethyl-6-phenylphenanthridinium Chloride (**2**).

Synthesis and purification procedures were adapted from Bastos (9) and Graves, *et al.* [21]. Ethidium bromide (**1**), (551 mg, 1.40 mmoles) was dissolved in 50 ml of 0.1 *N* hydrochloric acid, cooled to 0°, and diazotized with sodium nitrite (973 mg, 14.10 mmoles) dissolved in 10 ml water (0°). After 10 minutes, sodium azide (999 mg, 15.38 mmoles) in 10 ml of water (0°) was added. The reaction was allowed to continue for 1 hour, and then the carbinol base was precipitated with sodium hydroxide. The filtered precipitate was redissolved in *p*H 4.5 water (adjusted with hydrochloric acid) and chromatographed by cation exchange chromatography. Only a small fraction of brownish material remained at the origin with the majority of the product, **2**, appearing bright fluorescent yellow-green and eluting rapidly at *p*H 4.0. Fractions with an absorption maximum at 432 nm were determined pure and then lyophilized for a quantitative yield.

3-Amino-8-azido-5-ethyl-6-phenylphenanthridinium Chloride (**3**).

The synthesis was adapted from the procedure of Graves, *et al.* [21]. Ethidium bromide (**1**) (548 mg, 1.40 mmoles) was dissolved in 50 ml of 0.1 *N* hydrochloric acid and was cooled to 0°. Sodium nitrite (98 mg, 1.42 mmoles) dissolved in 10 ml of water (0°) was added dropwise. After 3 minutes, 99 mg of sodium azide (1.52 mmoles) in 10 ml of water (0°) was added and the reaction was maintained at 0° overnight. The *p*H was then adjusted to 6.5 with sodium hydroxide and the reaction was chromatographed on cation exchange media. The first product eluted at *p*H 4.0 as a fluorescent yellow-green band and was identified as compound **2**, the 3,8-diazido-compound (50.7 mg, 0.12 mmole, 8.5% yield). The second product which eluted at *p*H 3.5 was compound **4**, not previously recognized. This isomer fluoresced brighter orange than the **3** isomer and constituted a minor product. Isomer **4** eluted slightly faster than compound **3**, and compound **3** is eluted at *p*H 3.5 continuous with the band of compound **4**. Fractions were taken and read spectrophotometrically. Only fractions with an absorption maximum of 462 nm were kept and lyophilized. These fractions (compound **3**) were essentially the last of the band to elute at *p*H 3.5, and the band had a dull red-orange fluorescence. Unreacted ethidium (**1**) eluted last at *p*H 3.0 and appeared as a bright red orange fluorescent band (52.0 mg, 0.132 mmole, 9%). A tarry residue (10%) remained uneluted at the top of the column. The yield for compound **3** was 218 mg, 0.52 mmole, 37%.

3-Azido-8-amino-5-ethyl-6-phenylphenanthridinium Chloride (**4**).

Ethidium bromide (**1**) (540 mg, 1.37 mmoles) was acetylated according to the procedure of Berg [23] by adding acetic anhydride (0.13 ml, 1.40 mmoles) and stirring at room temperature for 2-3 hours. A precipitate, which was a mixture of the mono- and diacetylated derivatives and unreacted ethidium was isolated by filtration. This precipitate was resuspended in 50 ml of 0.1 *N* hydrochloric acid and cooled to 0° in an ice bath. Diazotization with sodium nitrite (272 mg, 3.94 mmoles) in 10 ml of water at 0° was carried out. At the end of 5 minutes, excess nitrous acid was destroyed with sulfamic acid. Sodium azide (269 mg, 4.15 mmoles) in 10 ml of water (0°) was then added and the reaction proceeded overnight. Sodium hydroxide was added to precipitate the carbinol base, and the mixture was filtered. The filter cake was resuspended in 100 ml of 2 *N* hydrochloric acid and heated gently while stirring for 2 hours at 70°. All the material dissolved within 30 minutes and deacetylation was considered to be complete when the fluorescence of the solution changed from green to orange. The solution was cooled on ice and sodium hydroxide was added again to precipitate the carbinol base. The precipitate was dissolved in *p*H 3.0 water, and the *p*H of the solution was adjusted to 6.5. Purification was effected by cation exchange chromatography, with elution beginning at *p*H 4.0. Three bands could be detected under uv light; a yellow-green fluorescent band of compound **2** which eluted first but was not collected (~10%), an orange fluorescent band of the **4** isomer which eluted next and a red-orange fluorescence band of unreacted ethidium (**1**) which also was not collected (~10%). The orange band was eluted from the column at *p*H 3.5 in fractions and the absorption spectra were examined for each fraction. Fractions with an absorption maxima of 454 nm were dried to give the amine salt of **4** to yield 405 mg, (0.96 mmole), 70%.

8-Amino-5-ethyl-6-phenylphenanthridinium Chloride (**5**).

Ethidium bromide (**1**) (545 mg, 1.38 mmoles) was acetylated according to a modified Berg procedure [23] as described above for compound **4** and diazotization was as described for compound **4**, except that 1.0 ml (12.0 mmoles) of 48% hypophosphorous acid, was included in the reaction mixture. After 2 days at 0°, the unreacted sodium nitrite was destroyed with sulfamic acids. The carbinol base was precipitated with sodium hydroxide and filtered. The precipitate was hydrolyzed in boiling 2

N hydrochloric acid for 2 hours. Again the carbinol base was precipitated with sodium hydroxide and the filtered precipitate was dissolved in *p*H 3.0 water. This solution was then adjusted to *p*H 6.5 and chromatographed on cation exchange medium. Three bands were detected by visible and uv light. A yellow band, **9**, which fluoresced violet was eluted first at *p*H 4.0 and constituted 7%, (33 mg, 0.10 mmole). An orange band, **5**, which fluoresced orange eluted next at *p*H 3.5; and unreacted ethidium (**1**) was last to elute at *p*H 2.5 and constituted 57 mg (0.14 mmole, 10%). Fractions of **5** were taken and read spectrophotometrically to obtain pure samples of **5** with an absorption maximum at 430 nm. These fractions were lyophilized and gave 254 mg (0.74 mmole, 53% yield).

8-Azido-5-ethyl-6-phenylphenanthridinium Chloride (**6**).

The 8-amino-5-ethyl-6-phenylphenanthridinium chloride (**5**) was prepared as described above according to the procedure of Berg [23], and 103 mg (0.30 mmole) was dissolved in 20 ml of 0.1 *N* hydrochloric acid and cooled to 0°. Sodium nitrite (65 mg, 0.94 mmole) in 5 ml of water (0°) was added. After 3 minutes, sodium azide (70 mg, 1.01 mmoles) in 5 ml of water (0°) was also added and the reaction was allowed to proceed overnight. The *p*H of the reaction mixture was adjusted to 6.5 with sodium hydroxide and the reaction was placed on a cation exchange column and eluted with *p*H 4.5 water. The major product **6** fluoresced a pale green and provided a quantitative yield.

3-Amino-5-ethyl-6-phenylphenanthridinium Chloride (**7**).

Ethidium bromide (**1**) (555 mg, 1.41 mmoles) was dissolved in 100 ml of 0.1 *N* hydrochloric acid at 0° with hypophosphorous acid (1.0 ml of 48%, 12.0 mmoles) and diazotized with sodium nitrite (98 mg, 1.42 mmoles) in 10 ml of water (0°). The reaction which was allowed to proceed overnight, was then adjusted to *p*H 6.5 and was placed on a cation exchange column and eluted with *p*H 4.5 water. Three major bands separated: a pale yellow band **9** which fluoresced violet eluted first; an orange band which fluoresced yellow-orange contained both **5** and **7**, a mixture of the monoamino analogs, began eluting at *p*H 3.5. The 8-amino-isomer **5** eluted slightly ahead of the 3-amino-isomer **7** and constituted a smaller portion of the mixture. The orange band was collected as fractions and the absorbance was recorded. The fractions with absorption maxima below 437 nm were discarded, so that no pure 8-amino-analog **5** was obtained. The fractions with an absorption maximum at 437 nm were collected and lyophilized, and were determined to be 95% (by nmr, 3-amino-isomer **7**, yielding 115 mg, (0.34 mmole, 24%).

3-Azido-5-ethyl-6-phenylphenanthridinium Chloride (**8**).

The 3-amino-5-ethyl-6-phenylphenanthridinium chloride (**7**) was prepared as described above and 101 mg (0.29 mmole) was dissolved in 20 ml of 0.1 *N* hydrochloric acid at 0°. Sodium nitrite (69 mg, 1.00 mmole) in 5 ml of water (0°) was added dropwise while stirring. Sodium azide (70 mg, 1.08 mmoles) in 5 ml of water (0°) was added after 5 minutes and the mixture was stirred overnight. The reaction mixture was adjusted to *p*H 6 and chromatographed on a cation exchange column and eluted at *p*H 4.5. The major product, **8**, showed a bright green fluorescenc and gave a quantitative yield.

5-Ethyl-6-phenylphenanthridinium Chloride (**9**).

Ethidium bromide (**1**) (555 mg, 1.41 mmoles) in 40 ml of 0.1 *N* hydrochloric acid was diazotized at 0° with excess sodium nitrite [910 mg, 13.19 mmoles, in 10 ml of water (0°)]. Hypophosphorous acid (2.0 ml of 48%, 23.0 mmoles) was included in the reaction which proceded overnight. The excess nitrous acid was destroyed with sulfamic acid and the carbinol base was precipitated with sodium hydroxide. The filtered precipitate was redissolved in *p*H 3.5 water. The *p*H of the solution was readjusted to 6.5 and the products were chromatographed using cation exchange medium and eluting at *p*H 4.5. The major product **9** was pale yellow and fluroesced violet giving a quantitative yield.

Acknowledgments.

We wish to thank Mrs. Mary Burns for her help in preparing this manuscript and Dr. M. Geckle for his assistance in obtaining the nmr spectra.

REFERENCES AND NOTES

[1] This work was supported by NIH Grant AI 14808 (L. W. Y.) and U. A. B. Faculty Research Grant (C. L. W.).

[2] Department of Biochemistry, University of South Alabama, College of Medicine, Mobile, AL 36688, U.S.A.

[3] Chemistry Department, University of Alabama in Birmingham, University Station, Birmingham, AL 35294, U.S.A.

[4] Department of Chemistry, University of Rochester, River Campus Station, Rochester, NY 14627, U.S.A.

[5] Correspondence addressee.

[6] B. A. Cox, W. J. Firth, III, S. Hickman, F. B. Klotz, L. W. Yielding and K. L. Yielding, *J. Parasitol.*, **67**, 410 (1981).

[7] M. Fukunaga, L. W. Yielding, W. J. Firth, III and K. L. Yielding, *Mutat. Res.*, **78**, 151 (1980).

[8] L. W. Yielding and W. J. Firth, III, *ibid.*, **71**, 161 (1980).

[9] R. B. Bastos, *J. Biol. Chem.*, **250**, 7739 (1975).

[10] S. C. Hixon, W. E. White, Jr. and K. L. Yielding, *J. Mol. Biol.*, **92**, 319 (1975).

[11] L. W. Yielding, W. E. White, Jr. and K. L. Yielding, *Mutat. Res.*, **34**, 351 (1976).

[12] I. Zimmerman and H. W. Zimmermann, *Ber. Bunsenges. Physik, Chem.*, **81**, 81 (1977).

[13] C. G. Reinhardt and T. R. Krugh, *Biochemistry*, **17**, 4845 (1978).

[14] T. R. Krugh, F. N. Wittlin and S. P. Cramer, *Biopolymers*, **14**, 197 (1975).

[15] T. R. Krugh and C. G. Reinhardt, *J. Mol. Biol.*, **97**, 133 (1975).

[16] G. P. Kreishman, S. I. Chan and W. Bauer, *ibid.*, **61**, 45 (1971).

[17] G. Thomas and B. Roques, *FEBS Letters*, **26**, 169 (1972).

[18] B. L. Shapiro and L. E. Mohrmann, *J. Phys. Chem. Ref. Data*, **6**, 919 (1977).

[19] R. J. Abraham, "The Analysis of High Resolution NMR Spectra", Elsevier, New York, 1971, Chapter 3.

[20] H. Sternglanz, D. E. Graves, L. W. Yielding and C. E. Bugg, *J. Cryst. Mol. Struct.*, **8**, 93 (1978).

[21] D. E. Graves, L. W. Yielding, C. L. Watkins and K. L. Yielding, *Biochem. Biophys. Acta*, **479**, 98 (1977).

[22] D. E. Graves, C. L. Watkins and L. W. Yielding, *Biochemistry*, **20**, 1887 (1981).

[23] S. S. Berg, *J. Chem. Soc.*, 3635 (1963).