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Characterization of Pharmacologically Important Prototropic Species Derived from a Pyridinemethanol Antimalarial by Electronic Absorption and Fluorescence Spectroscopy

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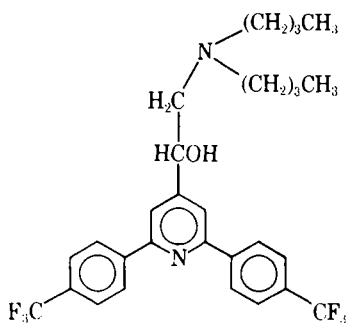
Abstract □ Variations of the absorption and fluorescence spectra of the experimental antimalarial drug, α-dibutylaminomethyl-2,6-bis(*p*-trifluoromethylphenyl)-4-pyridinemethanol, were investigated throughout the pH region in concentrated sulfuric acid media and in *n*-hexane. The predominant prototropic species at physiological pH is the singly charged cation. In the pH 6–12 region, the structured fluorescence of the monocation is quenched with the concomitant appearance of a diffuse, long wavelength emission while the corresponding absorption spectra shift only slightly to longer wavelengths. Furthermore, the dibutylamino group exhibits an unusually low basicity. This behavior is explained as due to the formation of an intramolecular hydrogen bond in the neutral molecule in the ground and lowest excited singlet states. A similar intramolecular hydrogen bond in the monocation is not spectroscopically visible.

Keyphrases □ Pyridinemethanol, substituted—prototropic species characterized from electronic absorption and fluorescence spectra at various pH values and in *n*-hexane □ Electronic absorption spectroscopy—used to characterize prototropic species of substituted pyridinemethanol at various pH values and in *n*-hexane □ Fluorescence spectroscopy—used to characterize prototropic species of substituted pyridinemethanol at various pH values and in *n*-hexane □ Antimalarial agents, potential—α-dibutylaminomethyl-2,6-bis(*p*-trifluoromethylphenyl)-4-pyridinemethanol, prototropic species characterized from electronic absorption and fluorescence spectra of various pH values and in *n*-hexane

A limited number of pyridinemethanol derivatives have been synthesized and screened for antimalarial activity (1–3). However, most of these compounds demonstrate either little activity or undesirable, toxic side effects (1). One of the few compounds of this class to exhibit antimalarial activity is the investigational drug α-dibutylaminomethyl-2,6-bis(trifluoromethylphenyl) -4- pyridinemethanol (I). It is 22 times more effective than quinine and is active against highly chloroquine-resistant *Plasmodium berghei* in mice (1). Although its specific mechanism of activity is unknown, it may be similar to, but not identical with, that of either chloroquine or quinine (1).



I

Most antimalarial compounds that exert their activity through a mechanism other than disruption of plasmodia nucleotide anabolism have at least one common feature. At physiological pH, these compounds exist as the singly charged cations or, as with chloroquine and quinacrine, as the doubly charged cation (4, 5). The cationic charge is believed to be involved in the binding of acridines, 9-aminoquinolines, and 8-aminoquinolines to parasitic DNA, and this binding is presumed to be related to antimalarial activity (6). Little is known about the mechanism of activity of quinine, alkaloids related to quinine, pyrimidines, quinolinemethanols, and pyridinemethanols. However, the singly charged cations of these agents may bind to plasmodia nuclear proteins, plasmodia DNA, or various enzymes in the metabolic network of these parasites (1, 7).

The present study of the dependence of the electronic spectra on the state of protonation and solvent properties of I was undertaken to elucidate the electronic structures of this compound and ultimately its interaction with native DNA, serum proteins, and selected enzymes.

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Table I—Electronic Absorption ($\lambda\,'L_b$ and $\lambda\,'L_a$) and Fluorescence (λ_f) Maxima of the Dication, Monocation, and Neutral Species of I in Aqueous Media [a]

	λ_a, nm		$\epsilon \times 10^{-3}$, liter/mole/cm	λ_f, nm
	$'L_a$	$'L_b$		
Dication	248 (0–0) 228 (max)	312 (max)	21	380 (max)
Monocation	238 (max)	296 (max) 286 (0–1)	8.5	348 (max)
Neutral species	246 (max)	305 (0–0) 290 (max)	6.5	478 (max)

[a] The dication was determined in 20% H_2SO_4 ($H_0 - 1.5$), the monocation was determined in dilute sulfuric acid at pH 3.0, and the neutral species was determined in dilute sodium hydroxide at pH 12.0. The molar absorptivities (ϵ) were determined at the long wavelength absorption maxima.

EXPERIMENTAL

Apparatus—Electronic absorption spectra were taken in 1-cm silica cells on a spectrophotometer[1]. Fluorescence spectra were recorded with a fluorescence spectrophotometer[2] having monochromators calibrated against the line emission spectrum of xenon. Emission and excitation spectra were corrected for the wavelength response of the light source, monochromators, beam splitter, and phototube by means of a variable resistor-corrected spectrum accessory utilizing an electric cam and rhodamine-B quantum counter. The pH measurements were made on a pH meter[3], employing a silver–silver chloride glass combination pH electrode[4].

Reagents—Compound I was supplied[5] as the hydrochloride salt. Elemental analysis of C,H,Cl,F,N yielded: calculated, 60.57, 5.78, 6.17, 19.82, 4.87; and found: 60.41, 5.62, 6.35, 19.61, 4.98. The melting point (234–235°) agreed with literature values (8, 9). The purity of the compound was verified by TLC in three different solvent systems: benzene–ethanol (9:1), R_f 0.70; chloroform–methanol (9:1), R_f 0.75; and ethanol–petroleum ether (bp 30–60°) (1:9), R_f 0.55.

All solvents were the highest grade commercially available. Acid solutions were prepared from reagent grade sulfuric acid[6] by dilution of the concentrated solution with distilled deionized water. Sodium hydroxide solutions were prepared by dilution, with distilled deionized water, of an 18 *M* solution prepared from USP grade sodium hydroxide pellets[6]. Reagent grade *n*-hexane[6] was used as necessary and did not interfere with the spectroscopic measurements.

Procedures—Ethanol solutions ($\sim 1 \times 10^{-3}$ *M*) of I were delivered from a 100-μl micropipet[7] into 100-ml volumetric flasks containing aqueous solutions at the desired pH. The added 0.1 ml caused negligible volume change. The ethanol solutions were too dilute to affect pH. All solutions were prepared immediately prior to use to remove decomposition errors and errors due to pH changes in poorly buffered solutions in the mid-pH region.

The corrected Hammett acidity scale of Jorgenson and Hartter (10) was employed to calibrate the sulfuric acid solutions. Solutions for the pH region were prepared by dilution of sulfuric acid or sodium hydroxide solution. Buffers were avoided because of their potential interference in fluorometric titrimetry (11). Trifluoroacetic acid was used to generate the dication in *n*-hexane. The hydrochloride salt was neutralized with 0.001 *N* NaOH to form the neutral species. The latter was extracted from solution with ether, allowed to dry, and then dissolved in *n*-hexane. All measurements were conducted at room temperature.

Ground- and excited-state dissociation constants, pKa and pKa*, respectively, were obtained graphically by plotting either the absorbance or relative fluorescence *versus* pH or Hammett acidity.

The ground-state pKa for the equilibrium between the monocation and neutral molecule was obtained potentiometrically. Ethanol solutions of I ($\sim 1 \times 10^{-3}$ *M*) as the hydrochloride salt were diluted with distilled, deionized water until their concentrations were 1×10^{-5} *M*. This procedure was necessary because of the extreme water insolubility of the compound. Three separate 100-ml aliquots of these solutions were titrated with 0.001 *N* NaOH, employing a microsyringe[8]. The pH was monitored during these titrations by the apparatus previously described. The pKa was determined graphically.

[1] Cary 118, Varian Associates, Instrument Division, Palo Alto, Calif.
[2] MPF-4, Perkin-Elmer Corp., Norwalk, Conn.
[3] Corning model 12, Fisher Scientific Co., Pittsburgh, Pa.
[4] Sensorex 5300C, Sensorex, Westminster, Calif.
[5] Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC 20012.
[6] J. T. Baker Chemical Co.
[7] Cole-Parmer Instrument Co., Chicago, Ill.
[8] Unimetrics Corp., Anaheim, Calif.

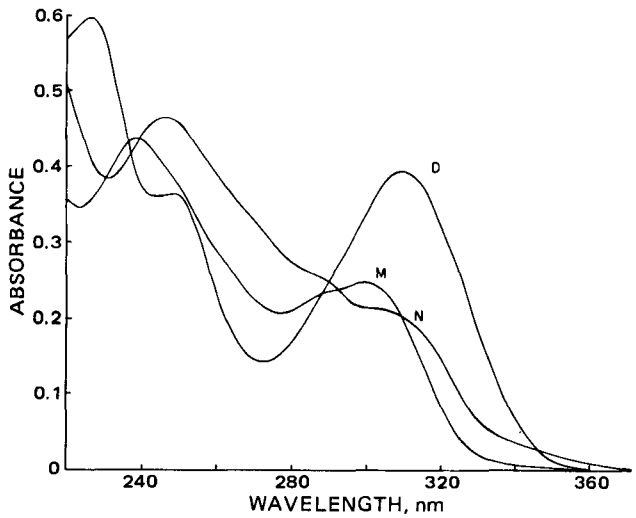


Figure 1—*Electronic absorption spectra of the dication (D), monocation (M), and neutral molecule (N) derived from I in aqueous and sulfuric acid media.*

RESULTS AND DISCUSSION

The electronic absorption and fluorescence maxima of the various prototropic species derived from I are presented in Table I, as are the molar absorptivities (ϵ) at the long wavelength absorption maxima of each species. The electronic absorption and fluorescence spectra of the dication, monocation, and neutral molecule are shown in Figs. 1 and 2, respectively.

For the dication, monocation, and neutral species, there are two distinct long wavelength absorption bands (Fig. 1). Earlier studies on 2-, 3-, and 4-hydroxypyridine derivatives related this two-band system, each band corresponding to a π–π^* transition, to that observed for various *ortho-*, *meta-*, and *para*-substituted benzene derivatives (12–14). The long wavelength band of the latter is the secondary band, while the band at shorter wavelengths is the primary band as designated by Doub and Vandenbelt (15, 16). These assignments correspond to the $'L_b$ and $'L_a$, respectively, in the notation of Klevens and Platt (17) and will be referred to as such here.

Acidity Dependence of Electronic Spectra—The pKa for the equilibrium between the singly charged and neutral species was deter-

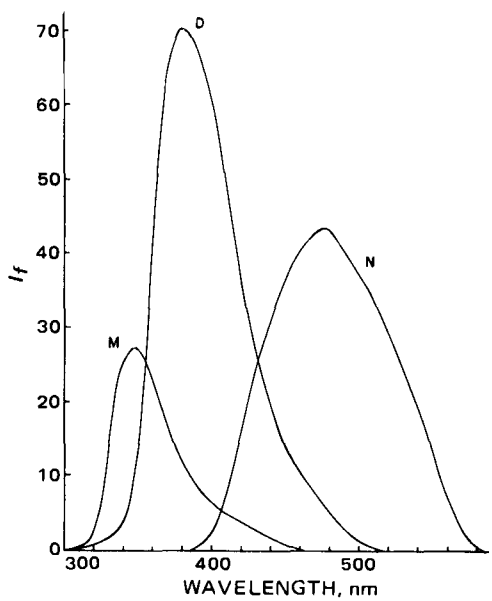
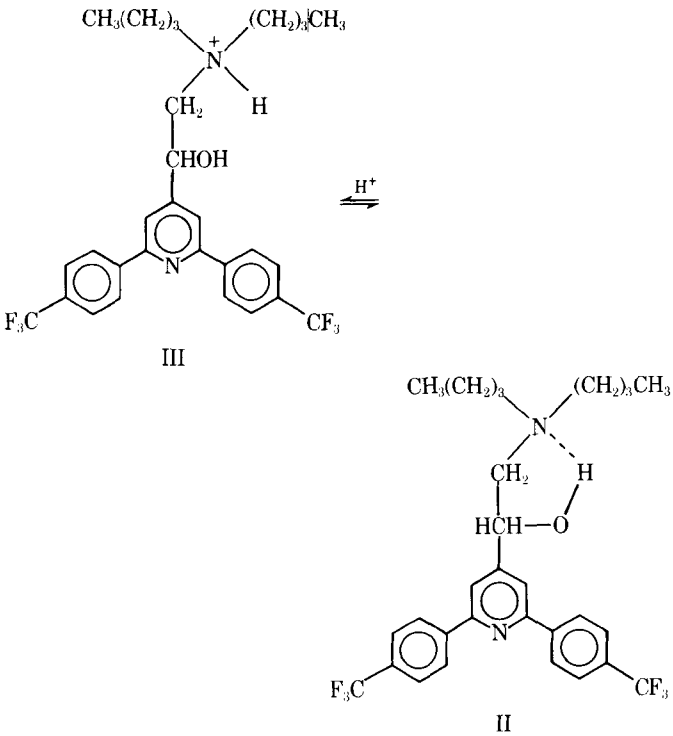


Figure 2—*Fluorescence spectra of the dication (D), monocation (M), and neutral molecule (N) derived from I in aqueous and sulfuric acid media; I_f is the relative fluorescence intensity.*



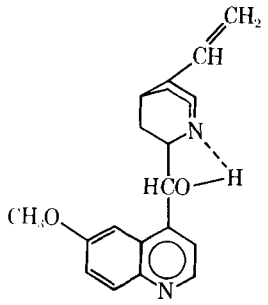
Scheme I

mined potentiometrically to be 8.3. Potentiometry was employed since the spectral band shape and position of the electronic absorption spectra of the monocation and neutral species were too similar to allow the spectroscopic determination of the ground-state dissociation constant. In the pH 3–14 region, the electronic absorption (both $'L_b$ and $'L_a$) and fluorescence maxima of solutions of I shifted to longer wavelengths (Figs. 1 and 2 and Table I) as solution pH was increased. The shift apparently corresponds to protonation of the neutral species, II, to form the monocation, III (Scheme I).

The pKa of the substituted exocyclic amino group relative to that of triethylamine (pKa 10.7) and tripropylamine (pKa 10.6) is anomalously low (18). Its decreased basicity may be the consequence of an intramolecular hydrogen bond between the hydroxyl hydrogen and the lone-pair electrons on the exocyclic nitrogen atom (Structure II). A similar decrease in the basicity of the quinuclidine nitrogen (pKa 8.4) in the neutral molecule of quinine was interpreted as due to an intramolecular hydrogen bond as shown in Structure IV (19). This interpretation also appears valid for II.

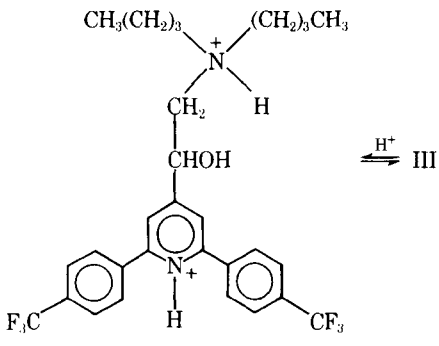
This interpretation may also be valid for the antimalarials mefloquine, a quinolinemethanol derivative (pKa 8.6), and 2,8-bis(trifluoromethyl)-4-[(1-hydroxy-3-*N*-*tert*-butylamino)propyl]quinoline[9] (pKa 8.22), which have 4-substituents capable of forming an intramolecular hydrogen bond and also exhibit unusually low pKa values for their exocyclic amino groups (20). Furthermore, it is supported by studies on the solvent dependence of the electronic spectra of all prototropic species derived from I and will be more fully discussed later.

Some differences in the electronic spectroscopy and chemistry of the two compounds (II and IV) can be attributed to the extent the postulated



IV

[9] WR 184,806.



Scheme II

five-membered ring in II interacts with the aromatic portion of the molecule. Of primary importance is the displacement of the electronic spectra of the neutral molecule of I to longer wavelengths relative to its conjugate acid, a phenomenon not observed in the neutral quinine molecule. The shift suggests an interaction of the five-membered ring with the extended pyridinic nucleus. It probably occurs through a stabilization of the neutral compound by hyperconjugation of the substituted carbinol carbon with the π-electron system of the aromatic nucleus. Delocalization of alkyl electrons may be enhanced by the substantial electron-withdrawing effect of the *p*-trifluoromethylphenyl groups in the 2- and 6-positions. The latter is sufficiently strong so that the electronic dipole moment of neutral I is similar to that observed in neutral quinine.

In the lowest excited singlet state, dissociation of the protonated exocyclic amino group occurs in more acidic solutions than when in the ground state (*e.g.*, $pKa = 8.3 \pm 0.1 > pKa^* = 5 \pm 1$). The corresponding prototropic reaction is not observed in quinine since the fluorometric titration follows ground-state titration characteristics. Evidently, the intramolecular hydrogen bond in II is much stronger in the lowest excited singlet state than when in the ground state, thereby reducing to a greater extent the availability of the lone-pair electrons on the exocyclic amino group for protonation. The latter, when considered with the large displacement of the fluorescence spectra, may suggest that the exocyclic amino group is involved in the donation of electronic charge to the aromatic nucleus, more so in the lowest excited singlet state than in the ground state.

Acidic solutions of I have their electronic absorption (both $'L_b$ and $'L_a$) and fluorescence spectra shifted to longer wavelengths as solution acidity is increased from pH 3.0 to $H_0 - 2$ (Figs. 1 and 2 and Table I). In the lowest excited singlet state, the singly charged species is a weaker acid than when in the ground state (*e.g.*, $pKa = 0.0 + 0.1 < pKa^* = 2.5 \pm 0.2$). Studies on aminopyridines and other nitrogen heterocycles suggest that this behavior indicates protonation of the heterocyclic ring nitrogen on III to form the dication V (Scheme II) (21–23).

The low ground-state dissociation constant for the equilibrium between III and V relative to that observed for the dissociation of the pyridinium ion (pKa 5.2) (24) is perhaps the consequence of two related factors. First, protonation of the heterocyclic nitrogen may be sterically hindered by the *p*-trifluoromethylphenyl substituents in the 2- and 6-positions. Studies on 1-phenylpyridine indicate that the phenyl group is coplanar with the pyridine nucleus, a result supported by studies on quinolinemethanol derivatives (1, 25). Moreover, 2,6-diisopropyl- and 2,6-di-*tert*-butylpyridine exhibit a marked steric hindrance to protonation (21). In view of these studies, it seems probable that each *p*-trifluoromethylphenyl group on I is coplanar with the pyridinic moiety, thereby hindering protonation of the heterocyclic nitrogen and contributing to the relatively low basicity of the pyridinic nitrogen. Second, the bandform and position of the wavelength maxima of the $'L_b$ band of the dication (Fig. 1) are similar to those observed for singly charged 2-phenylpyridine. Each of the latter bands lies between the long wavelength absorption bands ($'L_b$) for singly charged pyridine and quinoline (the monocations for each exhibits a λ_{max} at 255 and 330 nm, respectively) (26). Analogous to 2-phenylpyridine as well as biphenyl, the π-electrons of the pyridinic nucleus of I are probably delocalized on the *p*-trifluoromethylphenyl substituents. This configuration would also account for coplanarity of these groups with the pyridine nucleus and tend to deplete the heterocyclic ring nitrogen of electronic charge.

It is difficult to assess the electron-withdrawing inductive and π-electron delocalization effects of the *p*-trifluoromethylphenyl groups on the basicity of the heterocyclic ring nitrogen because of a scarcity of information. However, 2-phenyl substitution in pyridine has a base-

Table II—Long Wavelength Electronic Absorption (^1L_b) and Fluorescence (λ_f) Maxima of the Dication, Monocation, and Neutral Molecule of I in *n*-Hexane

	$\lambda\,^1L_b$, nm	λ_f, nm
Dication	318	385
Monocation	302	342 (0–0) 335 (max)
Neutral species	312	415

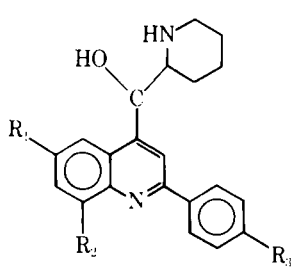
weakening effect of 1 log unit relative to pyridine; that of 2-*p*-nitrophenyl substitution is 1.6 log units (24, 25). Moreover, it is plausible to assume that 2-trifluoromethylphenyl substitution will have a base-weakening effect somewhat less than or very near the latter. Therefore, the substituents in the 2- and 6-positions on I cannot decrease basicity more than 3.2 log units. In the 4-position, the substituted carbinol carbon, which can only donate an electronic charge, may increase basicity by no more than 0.7 log unit. [For example, 2-methylpyridine has pKa 5.9. Delocalization of carbinol carbon electrons may be reduced by the electric field effect of the protonated exocyclic nitrogen, but this cannot be assessed at this time (24).] The overall base-weakening effect is approximately 2.5 log units relative to pyridine. Apparently, the low dissociation constant of doubly charged I is due equally to steric hindrance by the 2- and 6-substituents to protonation at the heterocyclic ring nitrogen and to the overall inductive effects and π-electron delocalization over the entire aromatic nucleus.

Solvent Dependence of Electronic Spectra—The Stokes shifts (absorption maxima to fluorescence maxima) of the neutral molecule in aqueous media (Table I) and *n*-hexane (Table II) are almost twice as large as those of the monocation and dication (Table III). Thermal relaxation processes from the Franck–Condon excited state to the thermally relaxed excited state are apparently greater in the neutral molecule than in either of the other species. A similar increase in the Stokes shift was interpreted by Weller (27) as an intramolecular proton transfer in the excited state. Since the hydrogen bonding in the five-membered ring of neutral I qualifies as partial proton transfer, the increase in the Stokes shift of the neutral molecule relative to the monocation and dication appears to be further evidence for the existence of the five-membered hydrogen-bonded substituent in the neutral species.

The shift of the electronic absorption spectra of all prototropic species to shorter wavelengths (increasing energy) upon changing the solvent from *n*-hexane to water suggests that the ground state of all species is stabilized relatively more than the Franck–Condon excited state and is, therefore, more polar (Tables I and II). The magnitude of the shifts for the dication, monocation, and neutral species (0.06, 0.07, and 0.17 μm^{-1}, respectively) suggests that the neutral species is stabilized greater than either the monocation or dication when in the ground state.

The corresponding shifts in the fluorescence spectra are to longer wavelengths (decreasing energy) upon increasing the polarity and hydrogen bonding capability of the solvent. The latter indicates that, in the lowest excited singlet state, all species are stabilized to a greater extent than when in the Franck–Condon ground state and are more polar. The values for the shifts of the dication, monocation, and neutral species are 0.03, 0.05, and 0.21 μm^{-1}, respectively. These values indicate that the neutral molecule also is stabilized to a greater extent than are the monocation and dication when in the lowest excited singlet state.

The greater stabilization of the neutral molecule in both ground and lowest excited singlet states may be due to the conformation change of the 4-substituent upon formation of a five-membered ring. This concept suggests a difference in conformation of the 4-substituent on the neutral and singly charged species, which may be due to a change in solvation of the latter. This notion is supported by the disparity in the Stokes shifts between the neutral molecule and the monocation (Table III). In fact, the similar magnitudes of these shifts for the monocation and dication



VI

Table III—Stokes Shifts Obtained from the Various Prototropic Species Derived from I in Aqueous Media and *n*-Hexane

	$\Delta\bar{\nu}$, μm^{-1}	
	Aqueous	*n*-Hexane
Dication	0.58	0.52
Monocation	0.62	0.39
Neutral species	1.2	0.80

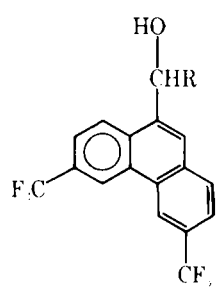
imply that the 4-substituent on these species is contained in the same solvent cage, which is different from that of the neutral molecule. Evidently, protonation of the neutral species at the exocyclic amino group disrupts the hydrogen-bonded five-membered ring. This rupture results in a reorientation of the solvent cage surrounding the 4-substituent such that the monocation does not engage in an intramolecular hydrogen bond that is observable by the methods employed in this study.

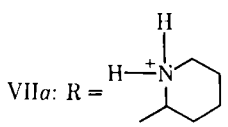
The relevance of the conformation of the 4-substituent in the monocation and neutral species is that a nonaromatic heterocycle, such as the piperidyl moiety in VI, may be necessary for the antimalarial activity of the amino alcohols. It was suggested (28) that amino alcohols similar to I, which possess an amino functional group substituted so that it is free to form an intramolecular hydrogen bond with the nearby carbinol hydroxyl group, owe their antimalarial activity, at least in part, to the formation of a hydrogen-bonded five- or six-membered nonplanar ring.

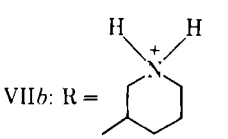
A more recent and specific study (29) dealt with the conformation of a family (VII*a*–VII*c*) of related antimalarial phenanthrene amino alcohols that have a methanolpiperidine substituent capable of forming an intramolecular hydrogen bond like the one observed in I and IV. The study proposed a model pharmacophore for the singly charged cation, which includes an intramolecular hydrogen bond between a hydrogen atom on the protonated piperidyl nitrogen in VII*a* and VII*b* and the carbinol oxygen atom. Structure VII*c* is said not to form such a hydrogen-bonded ring.

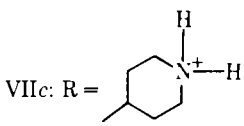
The results presented here for I indicate that the pseudo-oxazole ring system depicted by II is established and spectroscopically visible in the neutral species but not in the singly charged cation, III.

A possible explanation for these results is that the hydrogen bonding of the hydroxyl hydrogen atom with the exocyclic amino group decreases the steric hindrance of this atom with the hydrogens in the 3- and 5-positions on the pyridine ring. This situation could allow the pseudo-oxazole ring system to attain a greater degree of coplanarity with the pyridinic nucleus and thus account for the observed increase in electron delocalization. Protonation of the exocyclic nitrogen atom disrupts the hydrogen-bonded hydroxyl hydrogen from its association with the amino group. The possibility of mutual repulsion of the latter with the hydrogen atoms in either position 3 or 5 on the pyridinic nucleus is now increased and may be responsible for III not forming a ring system like that in II. The monocation, III, may form an out-of-plane hydrogen-bonded ring









similar to that reported for VII*a* and VII*b*; however, there is no definite spectroscopic evidence of its existence.

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Antitumor Agents XXVIII: Structural Elucidation of the Novel Antitumor Sesquiterpene Lactone, Microlenin, from *Helenium microcephalum*

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Abstract □ The structure and stereochemistry of the novel dimeric antitumor sesquiterpene lactone, microlenin, were determined on the basis of ^{1}H-NMR, ^{13}C-NMR, and mass spectral evidence.

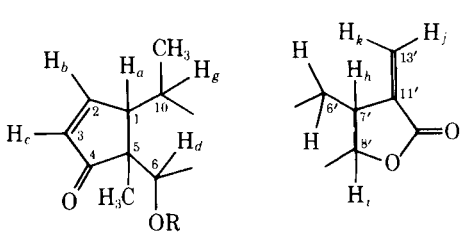
Keyphrases □ Microlenin—isolated from *Helenium microcephalum* whole plant extract, structure and stereochemistry determined □ *Helenium microcephalum*—whole plant extract, microlenin isolated, structure and stereochemistry determined □ Antineoplastic agents, potential—microlenin, isolated from *Helenium microcephalum* whole plant extract, structure and stereochemistry determined

A search for ample supplies of helenalin for the purpose of elucidating the structure–activity relationships between sesquiterpene lactones and antitumor activity led to the investigation of the plant *Helenium microcephalum*. Previously, it was reported (1) that this species contains helenalin in good yield. Examination of the whole plant extract revealed that, after the removal of helenalin, the mother liquor had constituents possessing significant inhibitory activity against the Walker 256 carcinosarcoma in rats. Further work with the chloroform extract resulted in the isolation and structure determination of mexicanin-E; microhelenins-A, B, and C (2, 3); and microlenin, a novel antineoplastic principle which resulted in a T/C value of 172 at 2.5 mg/kg/day in Walker 256 carcinosarcoma in Sprague–Dawley rats. The detailed structural determination of microlenin by physical methods is now reported.

RESULTS AND DISCUSSION

Microlenin (1*a*) was isolated by elution with chloroform–ethyl acetate from a silica gel column. Although the structure of microlenin was described previously (4), the stereochemistry and conformational assignments for microlenin require further comments. Microlenin contains an α,β-unsaturated cyclopentenone (IR bands at 1707 and 1580 cm^{-1}; ^{1}H-NMR: doublet of doublets of H_b and H_c at δ 7.84 and 6.02 ppm) and an α,β-unsaturated lactone of the type shown in partial structure A (IR bands at 1756 and 1664 cm^{-1}; narrowly split ^{1}H-NMR doublet of H_j and H_k at δ 6.13 and 5.73 ppm).

The presence of a secondary hydroxyl group (IR band at 3521 cm^{-1};



Partial Structure A

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