

# Role of the B-Ring Substituent in the Fluorescence of Colchicinoid-Tubulin and Allocolchicinoid-Tubulin Complexes<sup>†</sup>

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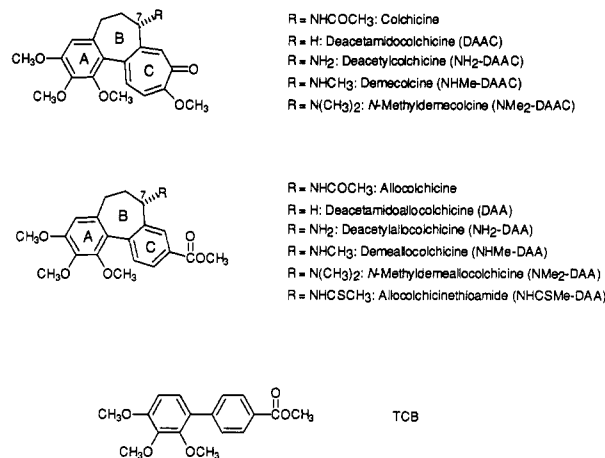
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**ABSTRACT:** Fluorescence spectra of several B-ring derivatives of allocolchicine in a variety of solvents have been obtained. The quantum yields of the allocolchicine derivatives decreased with amine substitution at the C-7 position [ $R = H > NH_2 \gg NHCH_3 > N(CH_3)_2$ ]. The relative fluorescent intensities of the aminoallocolchicinoids [ $R = NH_2, NHCH_3, N(CH_3)_2$ ] bound to tubulin were significantly less than the fluorescent intensities of tubulin-bound allocolchicine ( $R = NHCOCH_3$ ) and deacetamidoallocolchicine ( $R = H$ ). The low fluorescent intensities of the aminoallocolchicinoids in solvents and bound to tubulin could be explained by exciplex formation between the lone pair of electrons on the nitrogen and the allocolchicinoid  $\pi$ -system, which leads to quenching of the allocolchicinoid fluorescence. Direct evidence for exciplex formation between the C-7 amine nitrogen and the allocolchicinoid  $\pi$ -system was found in the emission spectrum of *N*-methyldemeallocolchicine [ $R = N(CH_3)_2$ ] in dioxane. The quantum yields of the aminoallocolchicinoids in glycerol were higher than would be predicted on the basis of solvent dielectric effects. Glycerol appears to increase the quantum yields of the aminoallocolchicinoids through both viscosity and hydrogen-bonding effects. The latter effect serves to sequester the lone pair of electrons on the nitrogen, decreasing its ability to interact with the  $\pi$ -system of the allocolchicinoid. It is concluded that the fluorescent properties of colchicinoids and allocolchicinoids in glycerol are not reliable indicators of the fluorescent properties of the molecules bound to tubulin. The relatively low quantum yields observed for aminocolchicinoids bound to tubulin are probably the result of intrinsic differences in the ligand fluorescent properties rather than a different binding site environment as was previously hypothesized [Bhattacharyya, B., Howard, R., Maity, S. N., Brossi, A., Sharma P. N., & Wolff, J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83, 2052-2055].

One of the characteristic properties of colchicine (Figure 1) in its complex with tubulin is the fluorescence of the tubulin-bound species. The mechanism by which induction of colchicine fluorescence upon tubulin binding occurs is not well understood. Colchicine has a low quantum yield in aqueous solution and in variety of organic solvents, yet tubulin binding promotes a dramatic ( $\sim 300$ -fold) increase in colchicine fluorescence (Bhattacharyya & Wolff, 1974; Arai & Okuyama, 1975). The sole condition found so far to partially mimic the fluorescence emission energy and quantum yield of tubulin-bound colchicine is increasing the rigidity of the molecule's environment, through freezing (Croteau & Leblanc, 1978), solvents such as glycerol (Bhattacharyya & Wolff, 1984), or inclusion into micelles (Shobha et al., 1989).

The system that has received the most study is colchicine fluorescence in glycerol. It was found that the emission intensity of colchicine is directly dependent on the viscosity of the solvent rather than the dielectric constant, and it has been proposed that tubulin binding serves to "immobilize" colchicine in a manner similar to increased viscosity (Bhattacharyya & Wolff, 1984). The fluorescence of colchicine and analogs in glycerol has therefore been used as a tool to investigate the fluorescent properties of other colchicine analogs.

The role of the B-ring substituent of colchicine in both the binding and subsequent fluorescent properties of the complex has received some debate. Recent thermodynamic studies of colchicine and analogs binding to tubulin suggest that there is no interaction between the B ring of colchicine and tubulin



**FIGURE 1:** Structures of colchicine, allocolchicine, and B-ring derivatives. Abbreviations for the molecules are based on C-7-substituted deacetamidoallocolchicine (DAAC) and deacetamidoallocolchicine (DAA).

in the equilibrium complex (Menendez et al., 1989; Andreu et al., 1991; Medrano et al., 1991). Yet the substituent at the C-7 position of the B ring seems to dictate the relative quantum yield of the colchicinoid-tubulin complex. Specifically, if the C-7 substituent is an amine or an alkyl amine, the quantum yield of the drug-tubulin complex is nearly zero, while colchicine analogs in which no substituent is present or the substituent is an acylated amine show the characteristic fluorescence enhancement (Bhattacharyya et al., 1986). All analogs tested have a similar quantum yield in glycerol, and the C-7 substituent is not in direct conjugation with the C ring. Thus it was concluded from the fluorescence analyses

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that the C-7 substituent on the B ring determines the quantum yield of the colchicinoid-tubulin complex, probably by changing the nature of the interaction of the molecule with tubulin.

We have recently investigated the spectroscopic properties of B-ring derivatives of colchicine in the presence and absence of tubulin (Pyles et al., 1992). The absorption and circular dichroic spectra of the tubulin-bound colchicinoids were similar regardless of whether the C-7 substituent was an acylated amine or an alkylated or free amine. We concluded that the binding-site environments of the colchicinoids, as probed by absorption and circular dichroic spectroscopy, are similar. Thus, the major spectral difference between the tubulin-bound ligands is manifested in fluorescence.

Through analysis of the absorption and circular dichroic spectra of the molecules in solvent, we noted that the nature of the C-7 substituent seemed to affect some electronic properties of the colchicine C ring, in spite of the absence of direct conjugation between the substituent and the C-ring  $\pi$ -system. It is therefore possible to hypothesize that the effect of the C-7 amine on the fluorescence of the ligand may indeed be primarily an intrinsic property of the molecule and not a consequence of its interaction with tubulin.

In this report, we have studied the effect of the C-7 substituent on the photochemical properties of alcolchicine derivatives. Alcolchicine rather than colchicine is chosen for the parent molecule because of the greater ease in studying the system. We have previously shown that the emission properties of alcolchicine can be explained by simple solvent effects and that the emission properties of the alcolchicine-tubulin complex are explicable by invoking a hydrophobic environment for the chromophore (Hastie, 1989). Our results with alcolchicine derivatives show that the nature of the C-7 substituent can dramatically affect the intrinsic fluorescence of the chromophore. By analogy, we propose that the relatively weak fluorescence observed when the C-7 amino derivatives of colchicine bind to tubulin is primarily the result of the presence of an efficient quencher in the vicinity of the fluorophore and not due to a differing interaction with the protein.

## EXPERIMENTAL PROCEDURES

**Materials.** Ethyl acetate, 2-propanol, methanol, dimethylformamide, ScintiVerse I, and triethylamine were purchased from Fisher Scientific. HPLC-grade acetonitrile was purchased from J. T. Baker and ethanol was obtained from AAPER Alcohol and Chemical Company. All other solvents and Lawesson's reagent were purchased from Aldrich. Dioxane, cyclohexane, methanol, glycerol, and dimethyl sulfoxide used for spectroscopy were spectrograde. Triethylamine, toluene, and methanol were dried over calcium hydride and distilled just prior to use. The other solvents used for spectroscopy were ACS certified. Pipes,<sup>1</sup> EGTA, MgSO<sub>4</sub>, and GTP (type II-S) were purchased from Sigma. [<sup>3</sup>H]Colchicine was from DuPont-New England Nuclear Research Products. Colchicine (U.S. Biochemical Corp.) was recrystallized from ethyl acetate prior to spectroscopic measurements.

<sup>1</sup> Abbreviations: Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMEG buffer, 0.1 M Pipes, 1 mM MgSO<sub>4</sub>, 2 mM EGTA, and 0.1 mM GTP, pH 6.90 at 23 °C; DMSO, dimethyl sulfoxide; TEA, triethylamine; MeCN, acetonitrile; TCB, 2,3,4-trimethoxy-4'-methoxycarbonyl-1,1'-biphenyl.

**Tubulin Purification and Protein Determination.** Bovine brain tubulin, free of microtubule-associated proteins, was prepared by two cycles of assembly/disassembly followed by phosphocellulose chromatography and frozen in liquid nitrogen (Williams & Lee, 1982). Tubulin obtained by this procedure is routinely >98% pure when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Prior to use, tubulin pellets were gently thawed, centrifuged at 800g for 10 min at 4 °C, and then desalted into PMEG buffer (0.1 M Pipes, 1 mM MgSO<sub>4</sub>, 2 mM EGTA, and 0.1 mM GTP, pH 6.90 at 23.0 °C) on 1-mL Sephadex G-50 columns according to the method of Penefsky (1977). Tubulin concentrations were determined spectrophotometrically by the use of an extinction coefficient at 278.5 nm of 1.23 (mg/mL)<sup>-1</sup> in PMEG buffer (Detrich & Williams, 1978). All experiments with colchicinoids and tubulin were performed in PMEG buffer. Experiments with alcolchicinoids were performed in PMEG buffer containing 10% DMSO (10% DMSO-PMEG). It has been previously determined that concentrations of up to 15% DMSO do not greatly affect ligand binding to tubulin (Head et al., 1985).

**Synthesis of Ligands.** Colchicine derivatives were prepared by literature procedures (Schreiber et al., 1961; van Tamelen et al., 1961; Capraro & Brossi, 1979). Structures and abbreviations for colchicine and alcolchicine analogs used in this study are shown in Figure 1. All alcolchicine derivatives except NH<sub>2</sub>-DAA and NHCSMe-DAA were synthesized by treating the appropriate colchicine analog with excess sodium methoxide (Fernholz, 1950). This procedure often led to the formation of carboxylic acid derivatives as well as esters. To convert all of the ring-contracted products to esters, the products from the sodium methoxide treatments were submitted to an ethereal diazomethane solution. NH<sub>2</sub>-DAA was synthesized by mild acid hydrolysis of alcolchicine and the product was submitted to methylation with ethereal diazomethane to give the methyl ester. NHCSMe-DAA was prepared by treating alcolchicine in dry toluene with 2.1 equiv of Lawesson's reagent (Fritz et al., 1978).

All colchicinoids and alcolchicinoids migrated as a single spot by TLC analysis. The compounds were characterized by <sup>1</sup>H NMR and <sup>13</sup>C NMR spectroscopy on a Bruker AM360 NMR spectrometer integrated to Aspect 3000 software, IR spectroscopy on a Nicolet 20SXC FT-IR with a 660 data station, absorption spectroscopy on a Hewlett-Packard Model 8451A diode array spectrometer, and by high- and low-resolution mass spectrometry on a VG 70-70 with an ionization voltage of 16 eV. Optical rotations were measured on a Perkin-Elmer Model 243B polarimeter. Melting points were measured on amorphous solids on a Sybron/Thermalyne melting point apparatus and were uncorrected.

Alcolchicinoid concentrations in 10% DMSO-PMEG solutions were determined spectrophotometrically using the following extinction coefficients:  $\epsilon_{290\text{nm}} = 1.53 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (alcolchicine; Hastie, 1989),  $\epsilon_{294\text{nm}} = 1.34 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (NMe<sub>2</sub>-DAA),  $\epsilon_{292\text{nm}} = 1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (NHMe-DAA),  $\epsilon_{290\text{nm}} = 1.38 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (NH<sub>2</sub>-DAA),  $\epsilon_{290\text{nm}} = 1.62 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (DAA),  $\epsilon_{270\text{nm}} = 1.63 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (NHCSMe-DAA), and  $\epsilon_{286\text{nm}} = 1.75 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (TCB). It is important to note that the DMSO must be added to the ligand before the buffer to ensure total solubility. Concentrations of colchicinoids in PMEG were also determined spectrophotometrically with the following extinction coefficients:  $\epsilon_{352\text{nm}} = 1.69 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (colchicine; Chabin et al., 1990),  $\epsilon_{356\text{nm}} = 1.45 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (NMe<sub>2</sub>-DAAC),  $\epsilon_{354\text{nm}} = 1.58 \times 10^4 \text{ M}^{-1}$  (NHMe-DAAC),  $\epsilon_{352\text{nm}} = 1.40 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$

(NH<sub>2</sub>-DAAC), and  $\epsilon_{352\text{nm}} = 1.62 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (DAAC).

**Fluorescence Spectroscopy.** Emission and excitation spectra were recorded on an SLM 8000 spectrophotometer operated in the ratio mode. The temperature was held constant with a circulating water bath at 25.5 °C except where noted. A 2 × 10 mm quartz fluorescence cell was used and oriented such that the excitation beam passed through the smaller path. Emission spectra were uncorrected and excitation spectra were corrected by a rhodamine solution in the reference channel. Solutions were made in the dark and at a concentration to avoid an inner filter effect. The spectrum of each fluorophore in solvent was measured at least two times. For the amino fluorophores (R = NH<sub>2</sub>, NHMe, and NMe<sub>2</sub>) in dioxane, ethyl acetate, DMF, and DMSO, fresh solutions were used for each spectrum in order to obtain reproducible results under steady-state conditions. Similar precautions have been taken for recording fluorescence spectra of amine-substituted hydrocarbons (Okada et al., 1977). New solutions were also used for the emission spectra of colchicine and NMe<sub>2</sub>-DAAC in dioxane. Emission spectra of allocolchicinoids and colchicinoids in solvent were recorded with excitation wavelengths of 290 and 330 nm, respectively. Data were collected at 1- or 2-nm intervals using an IBM PC interfaced to the instrument. Spectra of solvents were also collected and were subtracted from the appropriate spectrum using an IBM PC. Quantum yields of the allocolchicinoids in solvent were determined by comparison to the quantum yield of quinine sulfate in 0.1 M sulfuric acid (Demas & Crosby, 1971).

Allocolchicinoid-tubulin and colchicinoid-tubulin solutions were incubated at 37.0 °C for 60 and 90 min, respectively, just prior to recording of the excitation and emission spectra at 36.5 °C. The buffers used were 10% DMSO-PMEG (allocolchicinoids) and PMEG (colchicinoids). The concentration of tubulin in all solutions was 5.0 μM. Ligand concentrations were chosen such that quenching of intrinsic tubulin fluorescence, as measured from the maximum in the excitation spectrum when the emission wavelength was 320 nm (excitation wavelength = 260–310 nm), was the same for each allocolchicinoid or colchicinoid. Since quenching of tubulin fluorescence correlated with the occupancy of the binding site for allocolchicine (Medrano et al., 1989) and colchicine (Andreu & Timasheff, 1982), the concentration of tubulin-bound ligand was deemed to be equivalent in ligand-tubulin complexes prepared in this manner. The concentrations of the ligands required to quench tubulin fluorescence to the same extent were 15.0 μM DAA and DAAC; 15.1 μM colchicine; 10.0 μM allocolchicine; 30.0 μM NH<sub>2</sub>-DAA, NHMe-DAA, NH<sub>2</sub>-DAAC, NHMe-DAAC, and NMe<sub>2</sub>-DAAC; and 37.0 μM NMe<sub>2</sub>-DAA. Emission spectra of tubulin-bound species were recorded with an excitation wavelength of 350 nm (emission wavelength 370–600 nm) for the colchicinoids and 315 nm (emission wavelength = 330–570 nm) for allocolchicinoids bound to tubulin. The excitation wavelength for the allocolchicinoids bound to tubulin was chosen to avoid emission from tubulin (Hastie, 1989). Each solution containing fluorophore was run at least twice and the emission spectrum of the unbound ligand, which was indistinguishable from the emission spectrum of the buffer, was subtracted from the fluorophore's emission spectrum. The emission spectrum of each ligand was integrated, and the relative fluorescence of each ligand in its complex with tubulin was determined by comparing the area of each emission spectrum to that of the unsubstituted parent ligand (DAA or DAAC).

The effect of an added amine on the fluorescence of allocolchicine in acetonitrile was evaluated at 25.5 °C by

monitoring the emission intensity and the photochemical lifetime of allocolchicine as a function of triethylamine concentration. The allocolchicine concentration in all samples was 18.8 μM. The triethylamine concentration was varied between 0 and 717.5 mM. The emission spectrum of each solution was measured twice and was recorded immediately after sample preparation. The fluorescent intensity of each sample was determined at the emission maximum. The excitation wavelength for these spectra was 290 nm.

Fluorescence lifetimes of allocolchicine in the presence and absence of triethylamine in acetonitrile were determined by time-correlated pulsed single-photon counting. The samples were excited with light from a PRA Model 3000 510C gated nitrogen flash lamp which was controlled by a thyatron tube. The light was transmitted through an Instrument SA, Inc., H-10 monochromator, and the excitation wavelength was 290 nm. The light emitted from the sample was detected at 450 nm using a cooled Hamamatsu R955 photomultiplier tube. The photon counts were stored on a Tracer Northern Model 7200 microprocessor-based multichannel analyzer. The data are representations of the time-resolved decay which is distorted by the width of the lamp pulse. To correct for this distortion, a time profile of the excitation pulse was collected in a similar manner using a light scatterer in place of the sample. A diluted milk solution was used as the light scatterer. The instrument response function was deconvoluted from the fluorescence data and the corrected decay was then fitted by least-squares analysis on an IBM PC.  $\chi^2$  and Durbin-Watson statistical factors were used to determine the goodness of fit.

**Competition Experiments.** The relative fluorescence enhancement of the colchicinoids and allocolchicinoids bound to tubulin was determined by assuming that the occupancy of the binding site was directly proportional to the extent to which intrinsic tubulin fluorescence was quenched. This hypothesis was shown to be valid for the colchicinoids by a second set of experiments in which the ability of the colchicinoids to block tubulin binding of the bicyclic allocolchicine analog 2,3,4-trimethoxy-4'-methoxycarbonyl-1,1'-bi-phenyl (TCB) was evaluated. Briefly, tubulin (2.0 μM) was incubated with TCB (2.0 μM) and varying concentrations of the colchicinoid (colchicine, 10.0 μM; DAAC, 9.0 μM; NH<sub>2</sub>-DAAC, 48.0 μM; NHMe-DAAC, 33.9 μM; NMe<sub>2</sub>-DAAC, 45.3 μM) at 37.0 °C in DMSO-PMEG buffer (the concentration of DMSO ranged from 7 to 10%) and the emission intensity of the tubulin-bound TCB was recorded over time. The excitation and emission wavelengths were 315 and 385 nm, respectively. Under these conditions, tubulin-bound colchicinoid fluorescence is indistinguishable from the background. Concentrations of the colchicinoids were adjusted such that tubulin-bound TCB emission intensity at 385 nm was equivalent at equilibrium. Next, tubulin-colchicinoid complexes were formed using the colchicinoid concentrations determined from the experiments using TCB and the relative fluorescence of the colchicinoid bound to tubulin was determined (excitation wavelength = 350 nm and emission wavelength = 370–630 nm). This method gave results equivalent to the results obtained when quenching of intrinsic fluorescence was used to determine the occupancy of the colchicine site.

To demonstrate that quenching of intrinsic tubulin fluorescence is due to binding to the colchicine site and not to other regions of the protein, the effect of allocolchicinoids on the tubulin fluorescence of the colchicine-tubulin complex was examined. The colchicine-tubulin complex was formed by incubating 250 μM colchicine with 15 μM tubulin in 10%

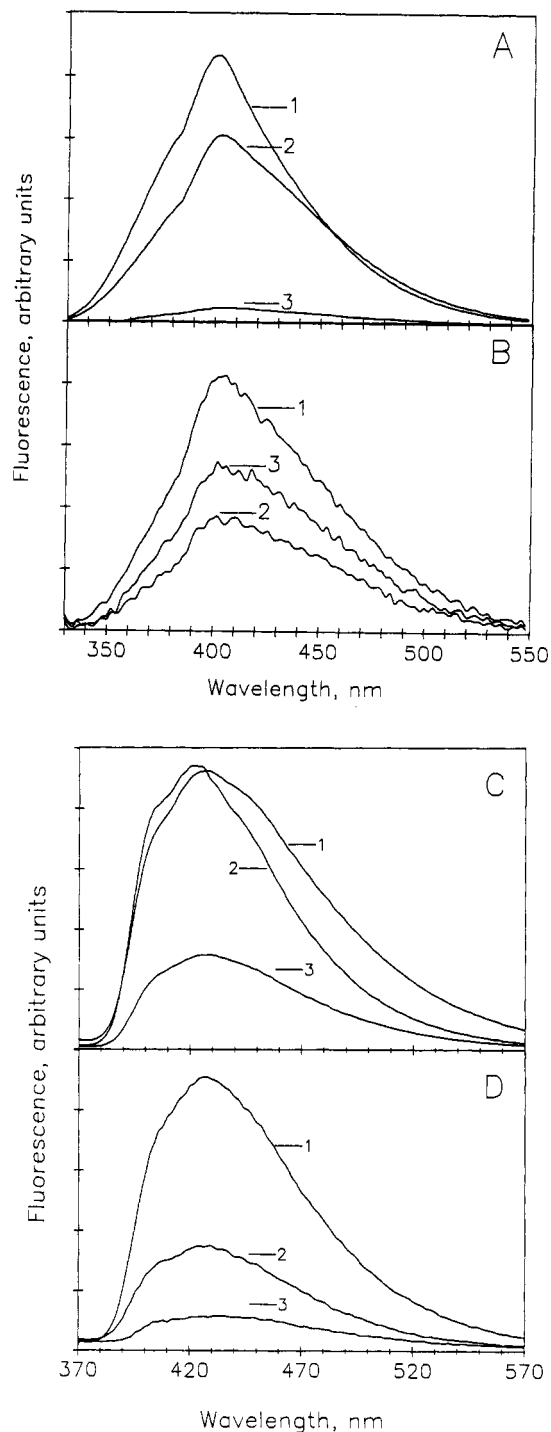
DMSO-PMEG for 30 min at 36.5 °C. Unbound ligand was removed from the complex by rapid gel filtration (Penefsky, 1977). The excitation spectrum from 260 to 310 nm (emission wavelength = 320 nm) of tubulin in the colchicine-tubulin complex (5.3  $\mu$ M) was measured in the presence and absence of allocolchicinoid ligands at the concentrations used in the enhancement experiments. In addition, one experiment was done by adding colchicine (38.1  $\mu$ M) to the colchicine-tubulin complex to account for any unoccupied colchicine binding site. The additional colchicine quenched the fluorescence of tubulin in the tubulin-colchicine complex to a very minor extent, and the added allocolchicinoids did not quench the fluorescence of the tubulin-colchicine complex relative to the added colchicine.

Inhibition of [ $^3$ H]colchicine binding to tubulin was performed as described previously (Hastie & Macdonald, 1990). Solutions containing [ $^3$ H]colchicine (5  $\mu$ M), tubulin (5  $\mu$ M), and the ligand to be tested were incubated at 37 °C in DMSO-PMEG (DMSO concentration ranged from 10 to 13%) for 90 min prior to separation of the unbound ligand. Allocolchicine, DAA, and NHCSMe-DAA inhibited [ $^3$ H]colchicine binding at low (5  $\mu$ M) concentrations (allocolchicine, 29%; DAA, 21%; NHCSMe-DAA, 49% inhibition). NHMe-DAA and NH<sub>2</sub>-DAA showed weak inhibition of [ $^3$ H]colchicine binding at higher concentrations (NHMe-DAA, 106  $\mu$ M, 5% inhibition; NH<sub>2</sub>-DAA, 106  $\mu$ M, 17% inhibition). Under the same conditions NMe<sub>2</sub>-DAA showed no inhibition of [ $^3$ H]-colchicine at concentrations up to 1 mM. Inhibition of [ $^3$ H]-colchicine was observed for NMe<sub>2</sub>-DAA (224  $\mu$ M, 8% inhibition; 1.05 mM, 48% inhibition) when the incubation time was decreased to 15 min.

## RESULTS

**Tubulin Binding of Allocolchicinoids.** Five allocolchicine derivatives that differ in the C-7 substituent were the primary focus of this study: allocolchicine, DAA, NH<sub>2</sub>-DAA, NHMe-DAA, and NMe<sub>2</sub>-DAA (Figure 1). The effect of tubulin binding on the fluorescence of the allocolchicine derivatives is shown in Figure 2, panels A and B. The concentrations of the allocolchicine analogs were adjusted such that all the molecules quenched intrinsic tubulin fluorescence to a similar extent and thus the extent of binding of the analogs can be considered similar in this figure. As in the analogous colchicine series (Figure 2, panels C and D), the two molecules in which the C-7 substituent was a hydrogen or an acetylated amine (DAA and allocolchicine) were strongly fluorescent in their complexes with tubulin, while the analog with an amine at the C-7 position was much less fluorescent (NH<sub>2</sub>-DAA) and the two alkylated amino derivatives (NHMe-DAA and NMe<sub>2</sub>-DAA) were slightly less fluorescent than NH<sub>2</sub>-DAA.

The assumption that the occupancy of the colchicine binding site is proportional to the extent to which intrinsic protein fluorescence is quenched was tested by two sets of experiments. First, the ability of colchicinoids to inhibit tubulin binding of the bicyclic allocolchicine analog 2,3,4-timethoxy-4'-methoxycarbonyl-1,1'-biphenyl (TCB, Figure 1) was evaluated. TCB rather than colchicine was chosen for these experiments because TCB rapidly forms a fluorescent species by binding to the colchicine site on tubulin (Medrano et al., 1991) and because the emission spectrum of tubulin-bound TCB can be observed in the presence of tubulin-bound colchicine without interference by colchicine fluorescence (unpublished observation). The concentrations of the colchicinoids required to inhibit TCB fluorescence to an equal extent were essentially the same as the concentrations required to quench intrinsic



**FIGURE 2:** Emission spectra of allocolchicinoids bound to tubulin (A and B) in 10% DMSO-PMEG and colchicinoids bound to tubulin (C and D) in PMEG. Ligand-tubulin complexes were prepared as described under Experimental Procedures. The integrated emission spectra of the ligands bound to tubulin are expressed relative to the unsubstituted parent ligands (DAA or DAAC). (A) Curve 1, DAA (100%); curve 2, allocolchicine (79%); curve 3, NH<sub>2</sub>-DAA (7%). (B) Curve 1, NH<sub>2</sub>-DAA (7%); curve 2, NHMe-DAA (4%); curve 3, NMe<sub>2</sub>-DAA (5%). (C) Curve 1, colchicine (122%); curve 2, DAAC (100%); curve 3, NH<sub>2</sub>-DAAC (38%). (D) Curve 1, NH<sub>2</sub>-DAAC (38%); curve 2, NHMe-DAAC (15%); curve 3, NMe<sub>2</sub>-DAAC (6%). The emission spectra of the unbound ligands, which are indistinguishable from the spectrum of the buffer, were subtracted from these spectra. The excitation wavelengths were 315 and 350 nm for the allocolchicinoid-tubulin and colchicinoid-tubulin complexes, respectively. The temperature was 36.5 °C.

tubulin fluorescence to an equal extent. Thus, inhibition of TCB binding and quenching of tubulin fluorescence provided similar results. Second, to demonstrate that quenching of

protein fluorescence was due to allocolchicinoid binding to the colchicine site on tubulin and not to other regions of the protein, the effect of allocolchicinoids on the tubulin fluorescence of the tubulin-colchicine complex was examined. Addition of allocolchicinoids to the tubulin-colchicine complex did not quench tubulin emission to an extent greater than colchicine itself.

We also attempted to determine whether the allocolchicinoids would inhibit [ $^3\text{H}$ ]colchicine binding to tubulin. Both allocolchicine and DAA strongly inhibited [ $^3\text{H}$ ]colchicine binding to tubulin (see Experimental Procedures). For the aminoallocolchicinoids ( $\text{NH}_2\text{-DAA}$ ,  $\text{NHMe-DAA}$ , and  $\text{NMe}_2\text{-DAA}$ ), however, the inhibition of [ $^3\text{H}$ ]colchicine binding was dependent on the length of the incubation. Specifically,  $\text{NHMe-DAA}$  and  $\text{NH}_2\text{-DAA}$  showed weak inhibition of [ $^3\text{H}$ ]colchicine binding when the samples were incubated for 90 min prior to evaluation of the amount of [ $^3\text{H}$ ]colchicine bound to tubulin. Inhibition of [ $^3\text{H}$ ]colchicine binding by  $\text{NMe}_2\text{-DAA}$  was not observed with a 90-min incubation, but slight inhibition could be seen when the incubation time was decreased to 15 min. These results indicate that the tubulin association properties of the aminoallocolchicinoids are different from those of allocolchicine and DAA. A knowledge of the equilibrium and kinetic parameters of the association of these compounds with tubulin would more precisely describe the nature of the interaction and assist in explaining the results obtained in the [ $^3\text{H}$ ]colchicine experiments. These determinations are beyond the scope of the present investigation.

**Fluorescence of Allocolchicinoids.** It is well known that aliphatic and aromatic amines can interact with excited states of aromatic hydrocarbons through the formation of both intermolecular and intramolecular complexes (Froehlich & Wehry, 1976; Mataga, 1981). We therefore sought to determine the effect of an external amine on the fluorescent properties of allocolchicine.

Addition of triethylamine (TEA) to a solution of allocolchicine in acetonitrile resulted in a concentration-dependent quenching of allocolchicine fluorescence and a concomitant decrease in the photochemical lifetime of the molecule but no change in the emission maximum. The quenching could be analyzed by a Stern-Volmer plot, yielding a  $k_q$  of  $1.16 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$  (Figure 3).<sup>2</sup> Thus, it is not unreasonable to propose that the low quantum yields of amino- and *N*-alkylaminocolchicinoids and -allocolchicinoids bound to tubulin may be due to the presence of an efficient quencher in the vicinity of the  $\pi$ -system.

Studies of intermolecular quenching of aromatic fluorescence by aliphatic amines have shown that the efficiency of quenching is a function of the amount of alkyl substitution on the aliphatic amine. In general, trialkylamines are better quenchers than dialkylamines, which in turn quench to a greater extent than monoalkylamines (Froehlich & Wehry, 1976). We therefore examined the effect of the number of alkyl substituents on the relative fluorescence of allocolchicine derivatives. The allocolchicine derivative DAA, in which there is no substituent at the C-7 position, was used as a reference compound. The relative quantum yield of the allocolchicine analog decreased with amine substitution and increasing number of alkyl groups on the amine, i.e.,  $\text{DAA} > \text{NH}_2\text{-DAA} > \text{NHMe-DAA} > \text{NMe}_2\text{-DAA}$ , in all solvents examined (Table I). The fluorescent intensities of the ami-

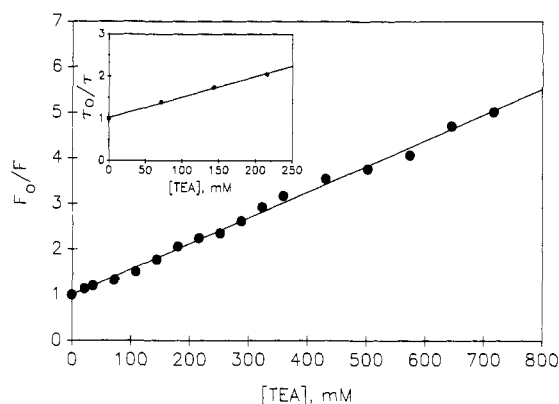


FIGURE 3: Triethylamine (TEA) quenching of allocolchicine fluorescence in acetonitrile as a function of TEA concentration. The fluorescent intensity of each solution (arbitrary units) was determined by measuring the fluorescent intensity at the emission maximum (448 nm). The concentration of allocolchicine was held constant at  $18.8 \mu\text{M}$ . TEA concentration was varied from 0 to  $717.5 \text{ mM}$ . The excitation wavelength was 290 nm and the temperature was  $25.5^\circ\text{C}$ . (Inset) Fluorescence lifetime of allocolchicine in acetonitrile as a function of TEA concentration.

Table I: Quantum Yields of Allocolchicinoids as a Function of Solvent

solvent	$E_{T30}^a$	DAA	$\text{NH}_2\text{-DAA}$	$\text{NHMe-DAA}$	$\text{N(Me)}_2\text{-DAA}$
dioxane	36.0	0.50	0.43	0.018	0.015
ethyl acetate	38.1	0.51	0.39	0.019	0.0084
dimethylformamide	43.8	0.47	0.24	0.010	0.0069
dimethyl sulfoxide	45.0	0.46	0.23	0.091	0.0075
acetonitrile	46.0	0.27	0.26	0.025	0.0049
2-propanol	48.6	0.23	0.095	0.049	0.0069
ethanol	51.9	0.054	0.037	0.023	0.0039
methanol	55.0	0.0092	0.0066	0.0072	0.0028
formamide	56.6	0.0088	0.0054	0.0053	0.0015
water	63.1	0.0006	<i>b</i>	0.0006	<i>b</i>
glycerol	57.0	0.053	0.047	0.045	0.024
tubulin <sup>c</sup>		100%	7%	4%	5%

<sup>a</sup> Values from Dimroth et al. (1963). <sup>b</sup> Too weak to be determined.

<sup>c</sup> Fluorescence emission of complex relative to that of DAA, determined as described under Experimental Procedures.

noallocolchicinoids bound to tubulin were substantially less than the fluorescent intensities of the DAA-tubulin complex (Table I) and the allocolchicine-tubulin complex (Figure 2). These results are consistent with the idea that the intrinsic fluorescence of the allocolchicine  $\pi$ -system is quenched by an amine at the C-7 position.

Quenching of aromatic fluorescence is believed to occur as a result of exciplex formation between the amine and the excited state of the aromatic chromophore (Froehlich & Wehry, 1976; Mataga, 1981). The next question that arises is whether an intramolecular complex between the C-7 nitrogen and the allocolchicine C ring may form. The distance between the amine nitrogen and the center of the aromatic C ring is  $\sim 3.5 \text{ \AA}$  (as measured from Dreiding models). Thus, it is conceivable that the quencher is close enough to the  $\pi$ -system to form a complex with the C ring. Direct evidence for such an interaction would be the observation of an exciplex in either the absorption or emission spectra of the allocolchicine derivatives. The low-energy absorption bands of all the allocolchicine derivatives (with the exception of allocolchicine itself) are virtually superimposable (data not shown), and it is impossible to determine whether an exciplex is formed by examination of the steady-state absorption spectra. Since exciplexes are very short-lived species, the absorption spectrum

<sup>2</sup> The  $k_q$  for TEA quenching of allocolchicine fluorescence is considerably less than expected for a diffusion-controlled reaction in acetonitrile ( $2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$  at  $25.0^\circ\text{C}$ ; Turro, 1978). This behavior is typical of aliphatic amine-aromatic hydrocarbons systems (Okada et al., 1976).

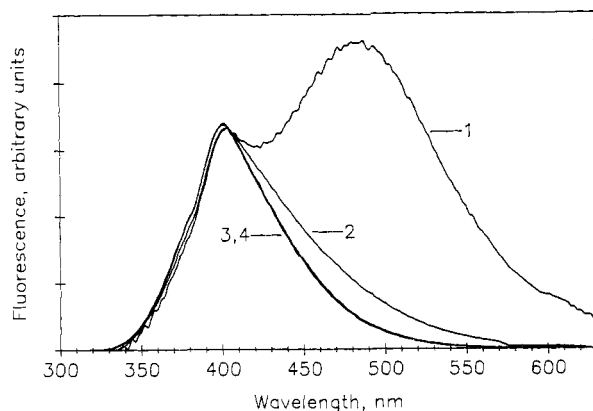


FIGURE 4: Emission spectra of  $\text{NMe}_2\text{-DAA}$  (curve 1),  $\text{NHMe-DAA}$  (curve 2),  $\text{NH}_2\text{-DAA}$  (curve 3), and  $\text{DAA}$  (curve 4) in dioxane. The spectra are normalized at the higher energy band (400 nm). The excitation wavelength was 290 nm and the temperature was 25.5 °C.

of an exciplex is not seen in the steady-state absorption spectrum (Froehlich & Wehry, 1976).

The emission spectra of the alcolcolchicine derivatives in dioxane as a function of the C-7 substituent do show evidence of exciplex formation (Figure 4). The emission spectrum of the monoalkylated derivative ( $\text{NHMe-DAA}$ ) is broadened in the low-energy region of the spectrum, while a second low-energy band clearly emerges in the dialkylated derivative ( $\text{NMe}_2\text{-DAA}$ ).

Thus it appears that the fluorescent properties of the alcolcolchicine derivatives as a function of solvent and tubulin binding can be explained on the basis of the intrinsic fluorescence of the ligand. Amino derivatives, which are capable of forming exciplexes with the alcolcolchicine  $\pi$ -system, are weakly fluorescent both in apolar solvents and bound to tubulin relative to alcolcolchicine derivatives in which the amine is acylated or in which no amine is present. The relative fluorescent intensity of the amino derivatives ( $\text{NH}_2\text{-DAA}$ ,  $\text{NHMe-DAA}$ , and  $\text{NMe}_2\text{-DAA}$ ) in solvent and when the ligands are bound to tubulin can be explained by the relative efficiency of the amine in quenching aromatic fluorescence.

To determine whether a substituent other than an amine would be able to act as an intramolecular quencher, we prepared an alcolcolchicine derivative in which the C-7 amide was replaced with a thioamide ( $\text{NHCSMe-DAA}$ , Figure 1). This compound was slightly more potent than alcolcolchicine in inhibiting [ $^3\text{H}$ ]colchicine binding to tubulin (see Experimental Procedures). Tubulin binding of  $\text{NHCSMe-DAA}$  resulted in a very minor enhancement of ligand fluorescence, which is consistent with the results obtained with the aminoalcolcolchicinoids.

The next question that arises is how these results can be reconciled with the previous studies of colchicinoids in glycerol. The observation that the B-ring substituent had little effect on the quantum yield of the molecules in glycerol indicated that there is no intrinsic reason for the molecules not to fluoresce to a similar extent when bound to tubulin (Bhat-tacharyya et al., 1986). It was concluded that the difference in the quantum yields of the colchicinoids bound to tubulin must be due to differing interactions with the protein.

We therefore examined the fluorescence of the alcolcolchicine derivatives in glycerol. The quantum yields for the molecules in glycerol are shown in Table I. Several points should be noted. First, with the exception of  $\text{NMe}_2\text{-DAA}$ , the quantum yields of all the alcolcolchicine derivatives in glycerol are essentially equivalent. Therefore, at least in the alcolcolchicine series, fluorescence of the ligand in glycerol

does not reliably predict the fluorescent intensity of the ligands bound to tubulin. Second, note the relative quantum yields of the molecules in glycerol as compared to dioxane. In the two compounds in which the C-7 substituent has little effect on the fluorescence of the compound ( $\text{DAA}$  and  $\text{NH}_2\text{-DAA}$ ), the quantum yield in glycerol is substantially lower than the quantum yield in dioxane. This is also true for alcolcolchicine ( $\phi = 0.044$  and 0.32 in glycerol and dioxane, respectively; Hastie, 1989). The relative fluorescent intensity of these three compounds could be explained in large part by solvent dielectric effects, with the viscosity of the medium being the reason that the quantum yields are somewhat higher than would be predicted on the basis of solvent dielectric constant. In the two compounds in which the C-7 substituent has a major effect on the quantum yields in dioxane ( $\text{NHMe-DAA}$  and  $\text{NMe}_2\text{-DAA}$ ), the quantum yields of the compounds in glycerol are significantly higher than would be predicted by solvent dielectric effects and in fact are greater than the quantum yields of the molecules in dioxane.

In glycerol, then, the effect of the C-7 amine (i.e., quenching) is reduced for the  $\text{NHMe-DAA}$  and  $\text{NMe}_2\text{-DAA}$  molecules. There are two possible origins for this effect: the increased viscosity of the medium could decrease the interaction of the amine with the aromatic  $\pi$ -system, or the glycerol could serve to sequester the lone pair of electrons on the amines through hydrogen bonding. Formation of heteroexcimers is inhibited by increased viscosity (Okada & Mataga, 1976), and in the fluorescent probes of the structure 2-(*N*-arylamino)-6-naphthalenesulfonate (ANS), exciplex formation is inhibited in glycerol due to viscosity effects (Kosower et al., 1975). It is noted that the relative fluorescence of  $\text{NHMe-DAA}$  and  $\text{NMe}_2\text{-DAA}$  in solvents capable of forming a hydrogen bond is generally greater than that of the other alcolcolchicine derivatives (see Table I), implying that the hydrogen-bonding nature of the solvent could be playing a role in the glycerol system.

Evidence in favor of a hydrogen-bonding interaction between the amine substituent and solvent is presented in Figure 5. Addition of small amounts of 2-propanol to  $\text{NMe}_2\text{-DAA}$  in dioxane caused a concentration-dependent decrease in both the intensity and wavelength maximum of the low-energy emission (exciplex) band (Figure 5A). The experiment was repeated using a solvent of similar dielectric constant, acetonitrile, rather than 2-propanol. When acetonitrile was added, the decrease in the intensity of the charge-transfer band was significantly less, and no wavelength shift occurred (Figure 5B).

We next sought to determine whether the results obtained from the alcolcolchicine analogs were applicable to the colchicine series. Colchicine itself is only weakly fluorescent in solvent, and the fluorescence is difficult to detect. Moreover, the colchicine C ring is known to be susceptible to photochemical rearrangements in solvent (Roigt & Leblanc, 1973); thus, great care was taken to minimize photochemical decomposition of the samples. The emission spectra of colchicine and  $\text{NMe}_2\text{-DAAC}$  in dioxane are shown in Figure 6. The emission spectrum of  $\text{NMe}_2\text{-DAAC}$  is less intense than that of colchicine and is also broadened at lower energy than the emission spectrum of colchicine. This result suggests that the conclusions drawn from the experiments with alcolcolchicine series may be applied to B-ring analogs of colchicine.

## DISCUSSION

The association of aliphatic and aromatic amines with the excited states of aromatic hydrocarbons has been extensively



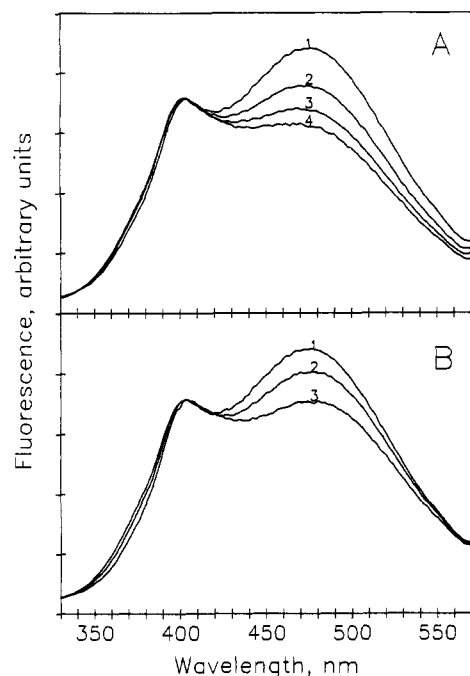


FIGURE 5: Effects of 2-propanol (A) and acetonitrile (B) on the emission spectrum of  $\text{NMe}_2$ -DAA in dioxane. All spectra are normalized at the higher energy band. The excitation wavelength was 290 nm. (A) Curve 1 is the spectrum of  $\text{NMe}_2$ -DAA in 100% dioxane. In curves 2, 3, and 4, the concentration of 2-propanol was 1%, 2%, and 3%, respectively. (B) Curve 1 is the spectrum of  $\text{NMe}_2$ -DAA in 100% dioxane. In curves 2 and 3, the concentration of acetonitrile was 1% and 3%, respectively. The concentration of  $\text{NMe}_2$ -DAA was 22.0  $\mu\text{M}$  in all spectra. The temperature was 25.5  $^\circ\text{C}$ .

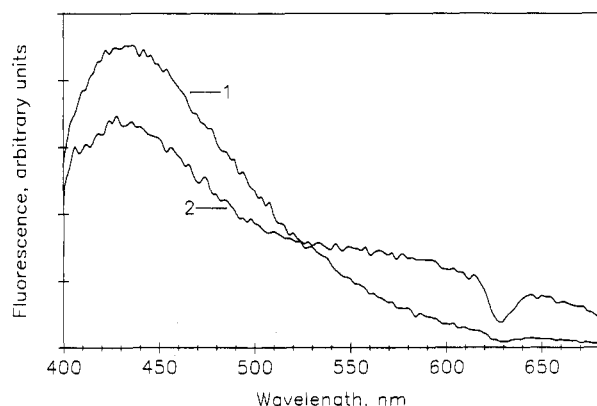


FIGURE 6: Emission spectra of colchicine (curve 1) and  $\text{NMe}_2$ -DAAC (curve 2) in dioxane. Great care was taken to ensure that minimal photodecomposition of the molecules took place during the experiment (see Experimental Procedures). The excitation wavelength was 330 nm and the temperature was 25.5  $^\circ\text{C}$ . The downturn in the spectrum at 640 nm is due to a Wood's anomaly, which is a property of the monochromator of the instrument (Jameson, 1984).

studied (Froehlich & Wehry, 1976; Mataga, 1981). The fluorescent properties of allocolchicine derivatives with varying amine substituents on the B ring in solvent are explicable in terms of exciplex formation between the C-7 amine and the aromatic portion of the molecule. The exciplex can be directly observed in the tertiary amine ( $\text{NMe}_2$ -DAA) in dioxane, but only quenching is observed in other solvents and in the primary and secondary amine derivatives ( $\text{NH}_2$ -DAA and  $\text{NHMe}$ -DAA). These observations are completely in agreement with previous work with aromatic hydrocarbons. Emission from exciplexes of tertiary amines is often quenched in polar solvents such that fluorophore-amine interactions appear to be a simple quenching process. Emission from exciplexes formed by

primary or secondary amines is generally not observed due to the presence of a hydrogen on the nitrogen atom. The exciplex formed in these systems may be deactivated through hydrogen abstraction by the aromatic ring, leading to photoproducts, as well as by emission of photons. Indeed, we have observed that the colchicine and allocolchicine derivatives in which the C-7 substituent is a primary and secondary amine are exceptionally prone to decomposition, even when stored at  $-20\text{ }^\circ\text{C}$  under an inert atmosphere in the dark. The purity of compounds of this nature must be monitored constantly.

The emission properties of allocolchicine bound to tubulin were previously analyzed in terms of allocolchicine emission in solvent (Hastie, 1989). The quantum yield and emission maximum of allocolchicine bound to tubulin were very similar to the quantum yield and emission maximum of the molecule in dioxane. In this work, we have shown that the emission properties of C-7 analogs of allocolchicine bound to tubulin are also described by the intrinsic fluorescent properties of the ligand. Allocolchicinoids that display a low quantum yield in dioxane form less fluorescent complexes with tubulin due to the presence of an efficient quencher in the vicinity of the fluorophore. By analogy and inferred from Figure 6, the fluorescent properties of B-ring analogs of colchicine can also be explained in terms of the chemical structure of the molecules. Thus, the differing spectroscopic properties of B-ring analogs of colchicine bound to tubulin do not necessarily reflect differing binding-site environments.

In both the colchicine and allocolchicine series, the fluorescent properties of the tubulin-bound species do not necessarily mimic the fluorescent properties of the molecules in glycerol. This observation does not negate the hypothesis that the origin of tubulin-bound fluorescence of colchicine is due, at least in part, to increased rigidity of the molecule's environment (Bhattacharyya & Wolff, 1984). The colchicine fluorophore (substituted tropone) has intrinsic photochemical properties different from the allocolchicine fluorophore (substituted biphenyl). What may be concluded from this work is that viscosity and hydrogen-bonding effects of glycerol are a function of both the nature of the fluorophore and the substitution pattern of the ligand and that the environment provided by glycerol does not necessarily emulate the environment of the colchicine binding site.

Whether the B ring and its substituents are indeed involved in the thermodynamic stability of colchicinoid-tubulin complexes cannot be determined from purely spectroscopic analyses. A clear picture of the role of the B ring in the thermodynamic stability of the colchicine-tubulin complex will require additional direct thermodynamic analyses of colchicinoids binding to tubulin.

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## SUPPLEMENTARY MATERIAL AVAILABLE

Detailed synthetic procedures and spectral data (mp,  $[\alpha]_D$ , IR, MS,  $^1\text{H}$  NMR, and  $^{13}\text{C}$  NMR) for the novel allocolchicinoids prepared for this paper (6 pages). Ordering information is given on any current masthead page.

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