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Effect of B-Ring Substituents on Absorption and Circular Dichroic Spectra of Colchicine Analogues†

Erica A. Pyles,‡ Richard P. Rava,*,§ and Susan Bane Hastie*,‡

Department of Chemistry, State University of New York, Binghamton, New York 13902-6000, and George R. Harrison Spectroscopy Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT: Near-ultraviolet absorption and circular dichroic spectra of several B-ring derivatives of colchicine have been obtained in a variety of solvents. The spectra of the molecules in solvent were analyzed and compared with spectra of the molecules bound to tubulin. Absorption spectra of deacetamidocolchicine, deacetylcolchicine, demecolcine, and *N*-methyldemecolcine [B-ring substituents = H, NH_2, $NHCH_3$, and $N(CH_3)_2$, respectively] were analyzed by multiple differentiation of the spectrum. It was found that an amine substituent at the C-7 position on the B-ring of the colchicinoid affected the higher energy transition of the near-ultraviolet spectra of the colchicinoid in the absence of tubulin in a manner consistent with a hyperconjugative alteration of this transition. The fourth derivatives of the absorption spectra of all four molecules bound to tubulin were similar to each other and to colchicine. As was true in the case of colchicine, the negative near-ultraviolet circular dichroic band of the aminocolchicinoids was relatively unaffected by solvent, but the molar ellipticity of the band was greatly reduced with tubulin binding. It is concluded that the binding site environments of the B-ring analogues of colchicine, as probed by absorption and circular dichroic spectroscopy, are equivalent.

Colchicine is a potent antimitotic agent that acts by binding strongly to the protein tubulin [for reviews, see Luduena (1979), Brossi et al. (1988), and Hamel (1990)]. Tubulin binding induces alterations in the absorption, fluorescence, and circular dichroic (CD)[1] spectra of colchicine that have not been precisely mimicked in the absence of the protein (Bhattacharyya & Wolff, 1974; Detrich et al., 1981). For example, colchicine binding to tubulin is accompanied by a dramatic increase in colchicine fluorescence when the tubulin–colchicine

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* To whom correspondence should be addressed.
‡ State University of New York, Binghamton.
§ MIT.

[1] Abbreviations: CD, circular dichroism; UV, ultraviolet; DAAC, deacetamidocolchicine; NH_2-DAAC, deacetylcolchicine; NHMe-DAAC, demecolcine; NMe_2-DAAC, *N*-methyldemecolcine; DMF, dimethylformamide; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMEG buffer, 0.1 M Pipes, 1.0 mM $MgSO_4$, 2.0 mM EGTA, and 0.1 mM GTP, pH 6.90.

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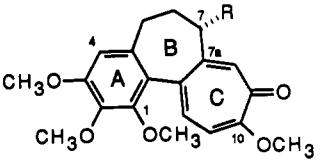
complex is excited in the near-UV band of colchicine (Bhattacharyya & Wolff, 1974). The energy and magnitude of tubulin-bound emission has not been duplicated in nonviscous organic solvents but can be partially emulated by increasing the rigidity of the molecule's environment through freezing (Croteau & Leblanc, 1978), solvents such as glycerol (Bhattacharyya & Wolff, 1984), or inclusion into micelles (Shobha et al., 1989). These types of analyses have led to the conclusion that tubulin binding serves to "immobilize" colchicine in a manner similar to increased viscosity (Bhattacharyya & Wolff, 1984).

Evaluation of the low-energy electronic spectra of tubulin-bound colchicine and analogues has been quite useful in probing the mechanism of how colchicine and related structures interact with tubulin [see, for example, Bhattacharyya and Wolff (1974), Detrich et al. (1981), Andreu and Timasheff (1982), Bhattacharyya et al. (1986), Hastie and Rava (1989), and Chabin et al. (1990)]. In some instances, however, conclusions made from spectroscopic analyses do not correlate with conclusions drawn from other techniques. Thermodynamic studies of colchicine and selected analogues binding to tubulin have led to the conclusion that the B-ring of colchicine is not involved in the thermodynamic stability of the colchicinoid–tubulin complex (Menendez et al., 1989; Andreu et al., 1991; Medrano et al., 1991). It has been shown, however, that the substituents on the B-ring affect the nature of the colchicinoid–tubulin fluorescence (Ray et al., 1981; Bhattacharyya et al., 1986). Specifically, if the C-7 substituent is an alkylated or free amine, the quantum yield of the complex is very low or essentially zero, while colchicine derivatives with no B-ring substituent or an acylated amine show the fluorescence enhancement characteristic of colchicine bound to tubulin. The differing fluorescence characteristics of the colchicinoid–tubulin complexes appear unrelated to the intrinsic fluorescence of the ligand, as all ligands fluoresce to a similar extent in glycerol. It was proposed that the *N*-alkylated derivatives may be interacting with tubulin in a manner different than the *N*-acylated analogues (Bhattacharyya et al., 1986).

We wished to ascertain whether differences in the fluorescent properties of the colchicinoid–tubulin complexes would also be manifested in the other spectroscopic properties of the low-energy electronic states. We have therefore examined the effect of tubulin binding on the near-UV absorption and CD spectra of deacetylcolchicine (NH_2-DAAC), demecolcine (NMe-DAAC), and *N*-methyldemecolcine (NMe_2-DAAC), three B-ring analogues of colchicine that do not display the dramatic fluorescence enhancement upon tubulin binding which is characteristic of colchicine (Bhattacharyya et al., 1986) (see Figure 1 for structures). We have also examined the absorption spectrum of deacetamidocolchicine (DAAC) as a function of solvent and when bound to tubulin. DAAC was included in this study because it shows the characteristic enhancement of ligand fluorescence when bound to tubulin but possesses no substituent at the C-7 position of the B-ring. Our results indicate that the nature of the C-7 substituent can affect the intrinsic spectroscopic properties of the colchicine C-ring. We conclude that differences in the tubulin-bound absorption and CD spectra of colchicine and the four B-ring analogues of colchicine studied in this work may be explained by inherent differences in the electronic transitions of the ligands.

Experimental Procedures

Materials. Pipes, EGTA, and GTP (type II-S) were obtained from Sigma. ACS certified grade dimethylformamide



R = $NHCOCH_3$: Colchicine

R = H: Deacetamidocolchicine (DAAC)

R = NH_2: Deacetylcolchicine (NH_2-DAAC)

R = $NHCH_3$: Demecolcine (NHMe-DAAC)

R = $N(CH_3)_2$: *N*-Methyldemecolcine (NMe_2-DAAC)

FIGURE 1: Structures of colchicine and B-ring derivatives of colchicine. The abbreviations for the B-ring derivatives of colchicine are based on the parent molecule deacetamidocolchicine (DAAC).

was obtained from Aldrich. Spectrograde methanol was from Fisher. Colchicine was obtained from U.S. Biochemicals and was recrystallized from ethyl acetate prior to use. Colchicine derivatives were prepared from colchicine using literature procedures (Schreiber et al., 1961; van Tamelen et al., 1961; Capraro & Brossi, 1979) and were characterized by infrared and proton NMR spectroscopy and mass spectrometry. All compounds migrated as a single spot by thin layer chromatographic analysis. The purity of the colchicinoids was constantly checked by thin layer chromatography and proton NMR spectroscopy to ensure the integrity of the samples used for spectroscopy.

The near-UV absorption spectra of the colchicinoids in aqueous solution each displayed a single broad band. The absorption maxima and extinction coefficients of the compounds in aqueous solution were as follows: colchicine (ϵ_{352} = 1.69×10^4 M^{-1} cm^{-1}; Chabin et al., 1990), *N*-methyldemecolcine (ϵ_{356} = 1.45×10^4 M^{-1} cm^{-1}), demecolcine (ϵ_{354} = 1.58×10^4 M^{-1} cm^{-1}), deacetylcolchicine (ϵ_{352} = 1.40×10^4 M^{-1} cm^{-1}), and deacetamidocolchicine (ϵ_{352} = 1.62×10^4 M^{-1} cm^{-1}).

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 as described previously (Williams & Lee, 1982) and stored in liquid nitrogen. Prior to use, the frozen pellets were gently thawed, centrifuged at 800*g* for 10 min at 4 °C, and then desalted into PMEG buffer (0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EGTA, and 0.1 mM GTP, pH 6.90 at 23 °C). Tubulin concentrations were determined spectrophotometrically by the use of an extinction coefficient at 278.5 nm of 1.23 $(mg/mL)^{-1}$ in PMEG buffer (Detrich & Williams, 1978).

Absorption Spectroscopy. Near-UV absorption spectra were measured at ambient temperature using a Hewlett-Packard model 8451A diode array spectrometer. Digitization of the spectra at 2-nm intervals was carried out by the spectrometer, and the digitized data were input into an IBM PC-AT for differentiation. The numerical methods for obtaining the derivatives and the assignments of the transitions of the spectra are discussed in detail in a previous paper (Hastie & Rava, 1989).

Colchicinoid–tubulin complexes for absorption spectroscopy were formed by incubating the ligand (40 μM) and tubulin (40 μM) in PMEG buffer in the dark at 37 °C for 90 min. After the incubation period, the colchicinoid–tubulin complex was separated from unbound ligand according to the method of Penefsky (1977). The absorption spectrum of the bound species was measured within 15 min after the gel filtration step.

CD Spectroscopy. CD spectra were obtained on a Jasco model J-20 ORD/UV-5 spectropolarimeter equipped with a Sproul Scientific SS15-2CD modification. All spectra were taken at ambient temperature (24 °C). At least three scans were made of each sample, and a baseline was always recorded in duplicate. The data were digitized using Un-Plot-It (Silk Scientific, Inc., Orem, UT) connected to an IBM PC-AT and stored in the form of ASCII files for subsequent manipulation.

Colchicinoid–tubulin complexes were formed by incubating the ligand (40 μM) and tubulin (20 μM) in the dark for 90 min at 24.0 °C. Spectra were run of the ligands in PMEG buffer (40 μM) and of the complex before and after removal of unbound ligand. Unbound ligands were separated from bound complexes by the method of Penefsky (1977). The concentration of tubulin after gel filtration was assessed by treating a tubulin solution without added ligand to the same procedure and measuring the absorption of the effluent. The concentration of the tubulin-bound ligand after gel filtration was determined as previously described (Chabin et al., 1990). Briefly, absorption difference spectra of the colchicinoid in the presence and absence of tubulin were measured to find isosbestic point(s) in the difference spectrum. The concentrations of colchicinoid and tubulin used for the difference spectra were 30 and 20 μM, respectively. The concentration of the tubulin-bound ligand was then determined by measuring the absorption of the sample at the corresponding wavelength. Colchicine showed an isosbestic point at 342 nm ($\epsilon = 1.59 \times 10^4$ M^{-1} cm^{-1}); *N*-methyldemecolcine showed isosbestic points at 348 and 372 nm ($\epsilon = 1.43 \times 10^4$ M^{-1} cm^{-1} and $\epsilon = 1.17 \times 10^4$ M^{-1} cm^{-1}, respectively). The demecolcine difference spectrum showed isosbestic points at 342 and 364 nm ($\epsilon = 1.49 \times 10^4$ M^{-1} cm^{-1} and $\epsilon = 1.45 \times 10^4$ M^{-1} cm^{-1}, respectively), and the deacetylcolchicine difference spectrum had isosbestic points at 344 and 370 nm ($\epsilon = 1.35 \times 10^4$ M^{-1} cm^{-1} and $\epsilon = 1.10 \times 10^4$ M^{-1} cm^{-1}, respectively).

The absorption and CD spectra of the tubulin-bound ligands were virtually identical immediately after gel filtration and up to at least 1 h after separation from the free ligand. In addition, the absorption derivative spectra of the ligands to tubulin resembled the spectrum of colchicine bound to tubulin rather than that of the free ligands in aqueous solution. It was therefore concluded that no detectable dissociation of the tubulin–ligand complexes occurred during the spectroscopic analyses.

Results

Absorption Spectra. The near-UV spectrum of colchicine is a broad, featureless band that centers around 353 nm in water (Bhattacharyya & Wolff, 1974), and little information about the electronic nature of colchicine can be obtained from the normal absorption spectrum. When the spectrum is subjected to multiple differentiation, the underlying structure of the absorption band is revealed. We have previously performed an extensive analysis of the electronic properties of colchicine, which may be deduced from the second and fourth derivatives of the absorption spectrum coupled with molecular orbital calculations (Hastie & Rava, 1989). Our results showed that the near-UV absorption band of colchicine is actually composed of two π–π^* transitions, one found between 360 and 390 nm (HOMO to LUMO) and the other located between 325 and 350 nm (HOMO to LUMO+1; see Table I).

The transition energies are affected by the hydrogen bond donating ability of the solvent. The fourth derivative spectra can be used to determine the energies of the two transitions and show the effect of the environment on the energy differences between the ground and excited state. In the case of colchicine, hydrogen bond donating solvents caused the lower energy transition to blue-shift relative to non-hydrogen bond donating solvents, while the higher energy transition was invariant with solvent. The ratios of the intensities of the lower energy peak in each of the two transitions can also be calculated. Particularly, we noted that for colchicine this ratio was near 1.0 for non-hydrogen bond donating solvents and 2.0 or greater for hydrogen bond donating solvents.

Table I: Observed Fourth Derivative Absorption Bands of Colchicine and B-Ring Analogues

solvent and colchicinoid	wavelength maxima (nm) low[a]	wavelength maxima (nm) high[b]	intensity of low/high[c]
DMF			
DAAC	386, 370 (366 sh)[d]	342, 325	1.2
colchicine[e]	385, 367	341, 323	1.0
NH_2-DAAC	384, 365	344, 326	1.1
NHMe-DAAC	384, 365	345, 327	1.1
NMe_2-DAAC	388, 362	347, 329	1.2
methanol			
DAAC	380, 364	341, 325	2.8
colchicine[e]	380, 362	340, *f*	2.2
NH_2-DAAC	379, 363	340, *f*	3.6
NHMe-DAAC	379, 363	340, *f*	2.8
NMe_2-DAAC	383, 364	~340, *f*	*f*
tubulin			
DAAC	386, 364	346, *f*	3.7
colchicine[e]	386, 364	345, 328	3.3
NH_2-DAAC	386, 364	346, ~328	3.0
NHMe-DAAC	388, 364	347, 328	2.1
NMe_2-DAAC	389, 364	345, 332	3.7

[a] Wavelength maxima of the pair of bands of the lower energy transition. [b] Wavelength maxima of the pair of bands of the higher energy transition. [c] Relative intensity of the peaks in the fourth derivative of the lower energy bands of the two transitions. Peak hights are measured from the abscissa to the peak maximum. [d] sh = shoulder. [e] From Hastie and Rava (1989). [f] Too weak to be determined.

Colchicine binding to tubulin caused a red-shift in the lower energy transition, consistent with a non-hydrogen bonding environment for the colchicine-binding site. The higher energy transition also underwent a red-shift, and the intensity ratio was much larger than found for a non-hydrogen bonding environment. We hypothesized that the latter two effects were the result of a π-stacking interaction between colchicine and an aromatic amino acid in the binding site.

Figure 2 illustrates the fourth derivatives of the absorption spectra of deacetamidocolchicine (DAAC), colchicine, deacetylcolchicine (NH_2-DAAC), demecolcine (NHMe-DAAC), and *N*-methyldemecolcine (NMe_2-DAAC) in dimethylformamide (DMF), a representative nonhydrogen bond donating solvent. The two transitions of the near-UV band, designated as the higher (H) and lower (L) energy transitions, are labeled. Some additional structure is present in the fourth derivative spectra of NH_2-DAAC, NHMe-DAAC, and NMe_2-DAAC, which may be due to either additional electronic transitions or additional vibronic activity, but the overall appearance of the fourth derivative spectra of all five of the molecules in DMF is very similar.

The fourth derivative spectra of the colchicinoids were examined as a function of solvent. As was true in the case of colchicine, the solvent effects could be grouped by the hydrogen bond donating ability of the solvent. Table I summarizes fourth derivative data for the molecules in two representative solvents which have similar dielectric constants but different hydrogen bond donating capabilities: DMF (non-hydrogen bond donating) and methanol (hydrogen bond donating). Although small differences are apparent when colchicine and DAAC are compared, the overall spectral features of DAAC in both hydrogen bond and non-hydrogen bond donating

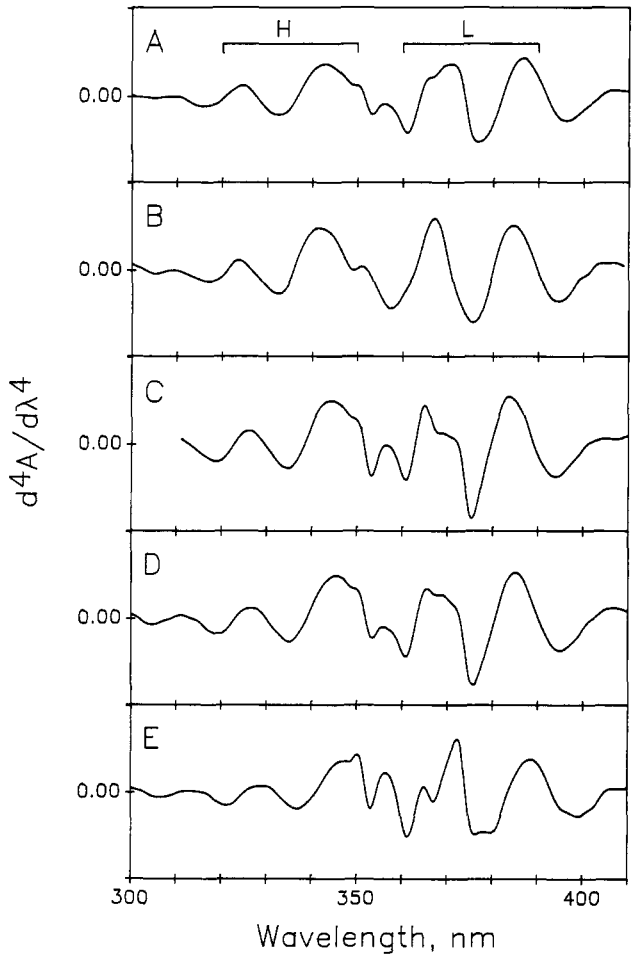


FIGURE 2: Fourth derivatives of the absorption spectra of DAAC (A), colchicine (B), NH_2-DAAC (C), NHMe-DAAC (D), and NMe_2-DAAC (E) in DMF. The absorption spectra of 40 μM solutions of the colchicinoids in DMF were numerically differentiated. The amplitudes of the derivative spectra are expressed in arbitrary units. Two bands of the high-energy transition (H) and low-energy transition (L) are designated by brackets.

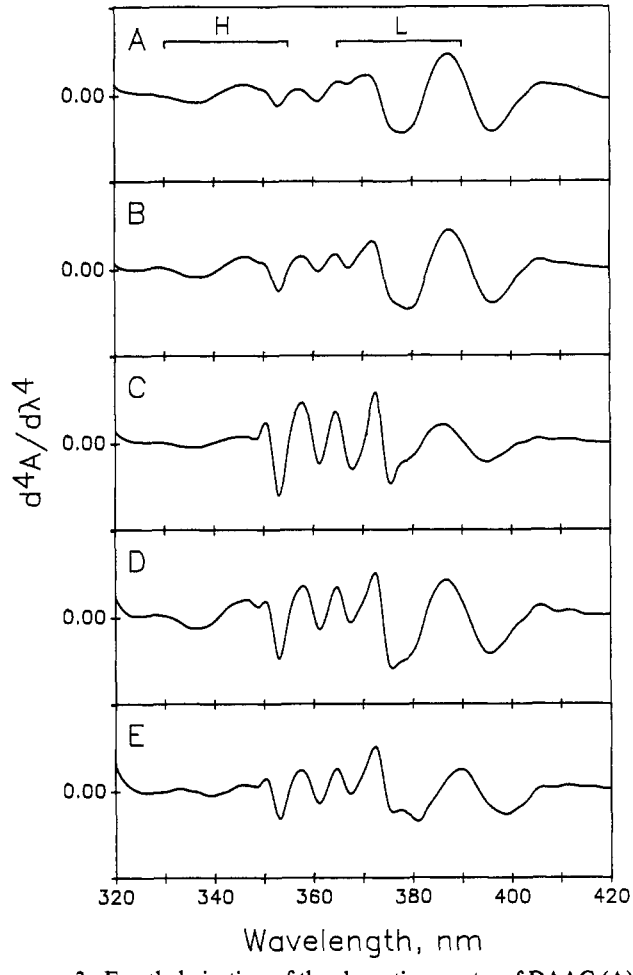


FIGURE 3: Fourth derivatives of the absorption spectra of DAAC (A), colchicine (B), NH_2-DAAC (C), NHMe-DAAC (D), and NMe_2-DAAC (E) bound to tubulin. The tubulin–colchicinoid samples were prepared as described under Experimental Procedures, and unbound ligand was removed prior to analysis. The amplitudes of the derivative spectra are expressed in arbitrary units.

solvents and when bound to tubulin are the same. Thus, it appears that the C-7 amide has little observable effect on the two low-energy electronic transitions of the C-ring.

The fourth derivative spectra of the three aminocolchicinoids, NH_2-DAAC, NHMe-DAAC, and NMe_2-DAAC, in solvent do not precisely follow the pattern set by colchicine. The characteristics of the lower energy transition and the intensity ratios of these three compounds follow colchicine and DAAC. The higher energy transition, however, becomes solvent dependent in the aminocolchicinoids (Table I). The amine substituent is therefore affecting the higher energy transition, possibly through an inductive or hyperconjugative effect. It is known that hyperconjugative effects perturb the energy of transitions while inductive effects perturb the intensities (Murrell, 1963). Since the relative intensities of the transitions seem to remain the same (as deduced from the intensity ratios of the transitions), the major effect of the amine as compared to the amide appears to be due to hyperconjugative perturbations.

The transition energies for the lower energy state of NH_2-DAAC, NHMe-DAAC, and NMe_2-DAAC in DMF and when the molecules are bound to tubulin are very similar, as seen in the fourth derivative absorption spectra of the aminocolchicinoids (Figure 3 and Table I). This is consistent with the results obtained for colchicine and DAAC. The transition energies for the higher energy state, however, shift to longer wavelength when going from DMF to tubulin for colchicine and DAAC but remain approximately the same for NH_2-DAAC, NHMe-DAAC, and NMe_2-DAAC. It is possible that the perturbation of the high-energy band by the amine substituent overshadows any additional wavelength shift such as the shift observed when colchicine and DAAC bind to tubulin. The intensity ratios in the fourth derivative spectra of all the colchicinoids bound to tubulin are similar, demonstrating that hyperchroism has occurred upon tubulin binding in all of the ligands. This latter result is consistent with a similar environment for all five colchicinoids involving an interaction with an aromatic amino acid in the binding site.

CD Spectra. CD spectra of colchicine, NH_2-DAAC, NHMe-DAAC, and NMe_2-DAAC in ethanol have been measured previously (Hrbek et al., 1982). It was noted that NH_2-DAAC had a small positive band at 410 nm, while no additional CD band was reported for the other colchicinoids. In contrast, we have found that in aqueous solution NMe_2-DAAC has a small positive band at 395 nm which is detectable at low concentrations and NHMe-DAAC has a very weak positive band around 395 nm which is only detected in solutions of high optical density. We have been unable to observe the reported 410-nm band in NH_2-DAAC in aqueous solution or in solvent. In working with these compounds, we have noted that the C-7 amine derivatives of colchicine are subject to decomposition, even when stored in the dark under an inert atmosphere. When a solution of NH_2-DAAC which contained

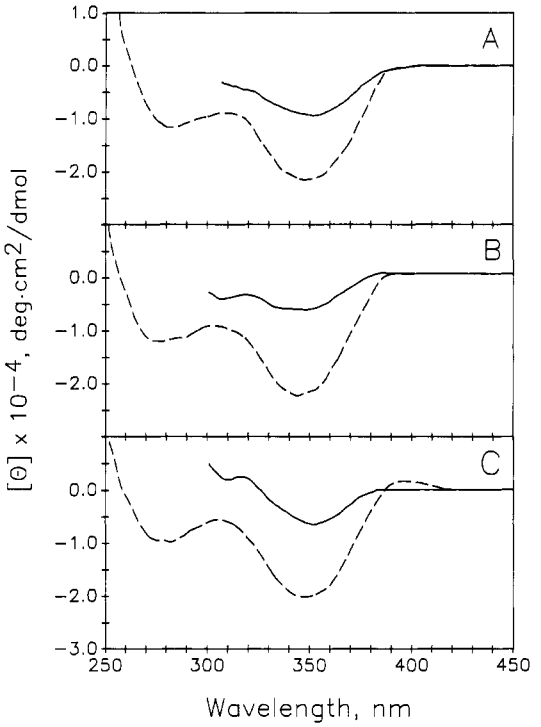


FIGURE 4: Near-UV CD spectra of NH_2-DAAC (A), NHMe-DAAC (B), and NMe_2-DAAC (C) in the presence and absence of tubulin. The dashed curve of each panel is the spectrum of the colchicinoid (40 μM) in PMEG buffer; the solid curve is the spectrum of each ligand bound to tubulin after the removal of unbound ligand. The samples were prepared as described under Experimental Procedures. The spectra are presented in molar ellipticity.

decomposed material was examined by CD, we observed a positive band at 415 nm in ethanol. Thus, we believe that the previous analysis of the CD spectrum of NH_2-DAAC was performed on an impure sample. Since the purity of the colchicinoids used in this study was rigorously monitored, the CD spectra presented here are deemed more correct than the earlier report.

The effect of tubulin binding on the negative near-UV CD band of NH_2-DAAC, NHMe-DAAC, and NMe_2-DAAC is shown in Figure 4. DAAC is a mixture of atropisomers which does not display an intrinsic CD spectrum (Yeh et al., 1988) and was therefore not studied by CD. As was true in the case of colchicine, each of the unbound molecules possesses a strong negative near-UV CD band corresponding to the low-energy transitions, and the characteristics of this band were essentially unaffected by solvent (spectra not shown). Upon tubulin binding, the molar ellipticity of the negative near-UV CD band decreases by ~70%, ~75%, and 60–70% for NMe_2-DAAC, NHMe-DAAC, and NH_2-DAAC, respectively. Under the same experimental conditions, colchicine undergoes an 80–85% reduction in intensity.

Discussion

Substituent Effects on the Electronic Spectra of Colchicinoids. Although the C-7 substituent is not in direct conjugation with the C-ring of colchicine, the substituent may affect the nature of the lowest energy transitions of the C-ring. We have found that if the C-7 substituent is $-NH_2$, $-NHCH_3$, or $-N(CH_3)_2$, the solvent dependence of the higher energy transition of the colchicinoid C-ring is altered relative to an amide or no substituent at this position. We have hypothesized that the effect may be due to hyperconjugation between the lone pair of electrons on the amine and the π-system of the C-ring. Hyperconjugative effects of amines on aromatic transitions are well documented (Murrell, 1963). For example, the low-energy transition of aniline is at much longer wavelength than benzene (Suzuki, 1967). At first inspection, it may be somewhat surprising that the C-7 amine, displaced as it is from the C-ring, could affect transitions in the tropone. In addition, it is curious that the amine seems to affect only the high-energy transition to a great extent. An explanation for this behavior may be found in the MNDO molecular orbitals calculated for colchicine (Hastie & Rava, 1989). We previously demonstrated that the higher energy transition is due to the HOMO to LUMO+1 excitation. Examination of the LUMO+1 orbital shows increased electron density at the C-7a carbon (numbered C8 in the paper), which would permit interactions of this excited state with the C-7 substituent on the B-ring. In contrast, the LUMO has virtually no electron density at this atom, which is consistent with the lack of an observed interaction in the low-energy band.

Evidence for an effect of an amine at the C-7 position and the tropone C-ring of colchicinoids is also seen by CD spectroscopy. The CD spectrum of NMe_2-DAAC shows an additional positive band at 395 nm, which may be associated with a charge-transfer transition from the amine to the tropone ring. Emission properties of charge-transfer complexes between amines and aromatic hydrocarbons have been extensively studied (Froehlich & Wehry, 1976; Mataga, 1981). The emission properties of charge-transfer complexes between amines and aromatic hydrocarbons differ depending on the number of alkyl substituents on the amine nitrogen (Froehlich & Wehry, 1976). Thus, it is not surprising that this apparent charge-transfer band would be more readily observable in the tertiary amine (NMe_2-DAAC) than in the primary and secondary amines (NH_2-DAAC and NHMe-DAAC).

Absorption and CD Spectra of B-Ring Colchicine Analogues Bound to Tubulin. The fourth derivative absorption spectra of all five colchicinoids bound to tubulin are essentially equivalent. The wavelength maxima and intensity ratios found for the tubulin-bound colchicinoids are explicable by invoking a π-stacking environment between the colchicinoid and an aromatic amino acid of tubulin (Hastie & Rava, 1989). The loss of optical activity upon tubulin binding, as measured from the molar ellipticity of the near-UV CD band, is not as great in the amine-containing derivatives as in colchicine itself, but otherwise the CD spectra of the aminocolchicinoids in solvent and bound to tubulin show behavior similar to that of colchicine. Thus, the CD spectra of the colchicinoids bound to tubulin are consistent with perturbation of the tropone by an aromatic moiety in the binding site as previously proposed (Chabin et al., 1990).

Conclusions

The three different spectroscopic techniques used to analyze the interaction of C-7 derivatives of colchicine each provide different types of information. Absorption spectroscopy primarily probes interactions occurring in the ground state. The data obtained from absorption spectroscopy support a binding site environment which is apolar (nonhydrogen bonding) and contains an aromatic amino acid residue. Fluorescence spectroscopy primarily detects interactions that affect the excited state. The previous fluorescence studies of these compounds demonstrate that the excited state environment is different for the *N*-alkylated amines vs amides (Bhattacharyya et al., 1986). Circular dichroic spectra are primarily affected by perturbations in the ground state as well as by the symmetry of the molecule and its environment. The CD spectra of the molecules bound to tubulin can be attributed to a stacking interaction between the C-ring of the molecule

and an aromatic amino acid in the binding site (Chabin et al., 1990). The small positive band in the spectrum of NMe_2-DAAC may be interpreted as an interaction between the ground state of the amine with the excited state of the tropone chromophore. This latter effect could be detected by fluorescence spectroscopy but not by steady-state absorption spectroscopy (Froehlich & Wehry, 1976). Thus, to truly understand the electronic properties of these complex systems, multiple techniques should be used.

Two major conclusions can be drawn from this work. First, the nature of the C-7 substituent can affect the electronic transitions of the tropone ring in colchicinoids. Thus, differences in the spectra of B-ring analogues bound to tubulin may be a result of intrinsic differences in the molecules themselves. Secondly, while the differences in the tubulin-bound fluorescence of NH_2-DAAC, NHMe-DAAC, and NMe_2-DAAC vs DAAC and colchicine were found to be very dramatic, the differences seen in the absorption and CD spectra of the tubulin-bound ligands are much more subtle. In terms of the absorption and CD spectra, then, there is little compelling reason to invoke a significantly different binding site environment for the five colchicinoids studied here. In light of the interaction observed between the C-7 amine and the electronic transitions of the tropone ring, it seemed fruitful to pursue the hypothesis that the differences in tubulin-bound ligand fluorescence are intrinsic to the ligand. The results from this investigation will be presented in a separate report.

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