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The importance of the phenyl-tropolone '*aS*' configuration in colchicine's binding to tubulin

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Measuring ellipticities of (±)-colchicine and (±)-deacetamidocolchicine in the presence of tubulin afforded net positive CD bands with maxima at 340 nm resulting from reduction of the negative ellipticities upon binding of (–) enantiomers to the protein. Results of optical studies together with earlier NMR conformational analysis of these molecules substantiate the hypothesis that colchicinoids bind to tubulin with the phenyl-tropolone moiety in the '*aS*' configuration. Natural colchicine which binds to tubulin, therefore, should be referred to as (–)-(*aS*,7*S*)-colchicine.

Colchicine; Tubulin; CD; Atropisomerism

1. INTRODUCTION

Colchicine, the major alkaloid from *Colchicum autumnale*, has antimitotic properties, and is used medicinally for the treatment of gout and Familial Mediterranean Fever [2–4]. Colchicine inhibits microtubule assembly both in vitro and in vivo by binding with high affinity to tubulin [5], a major protein subunit of microtubules. Although the results of antitumor activity and tubulin binding studies of a large number of cholchicinoids had indicated the importance of the spatial arrangement of the four methoxy groups in the drug-protein interaction, the mechanism of interaction is still not clearly understood. A recent report [6] which suggested that an *aS* configuration of the phenyl-tropolone moiety was essential for the interaction with the protein would therefore deserve special attention. Support for this hypothesis has now been obtained from circular dichroism (CD) measurements of natural (–)-(7*S*)-colchicine, unnatural (+)-(7*R*)-colchicine, (±)-colchicine and (±)-deacetamidocolchicine with and without addition of bovine brain tubulin.

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Note: The italic letter *a* before the corresponding *R* and *S* denotes an axial chirality originating from a biaryl system as suggested by Cahn et al. [1]

2. MATERIALS AND METHODS

Natural (–)-(7*S*)-colchicine was purchased from Fluka (Ronkonkoma, NY). Unnatural (+)-(7*R*)-colchicine was prepared as in [7]. Deacetamidocolchicine was made by catalytic reduction (Pd/C in ethyl acetate) of deacetamido-5, 6-dehydrocolchicine, prepared according to [8]. Deacetamidocolchicine, thus prepared, showed chemical and physical properties identical with material obtained by total synthesis [9]. All other chemicals were obtained commercially. Tubulin was purified from bovine brain using the published procedure [10] and stored in liquid nitrogen at a protein concentration of 54 mg/ml of 1 M sodium glutamate solution at pH 6.6 before use. Samples of tubulin for the CD experiments were prepared by rapidly thawing the stored material, followed by centrifugation, if needed to remove small amounts of denatured protein, and then diluting to a final protein concentration of approx. 2–2.5 mg/ml of buffer (1 M sodium glutamate, pH 6.6, 0.1 M α-D-glucose 1-phosphate, 1 mM $MgCl_2$ and 0.1 mM guanosine 3′-diphosphate). Concentrations of tubulin were determined according to Lowry et al. [11] or the method in [12] (The protein assay kits, purchased from Bio-Rad (Rockville Centre,

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NY), were used for measuring total protein concentration. The method is based on the Bradford dye-binding procedure [12].). Colchicine stock solutions were prepared by first dissolving colchicine in 0.5 ml methanol, then adding 0.5 ml buffer to obtain a final drug concentration of 2.2 mM. CD spectra were measured on a Jasco model J500A spectropolarimeter equipped with a data processing system for signal averaging. All spectra were taken at 24°C, using a 1 cm cell and a full-scale sensitivity of 40×10^{-3} degree. Ten scans were accumulated and averaged on all solutions. Aliquots of the drug stock solution were introduced into 3-ml samples of either buffer or tubulin solution in the CD cell. The CD spectra were measured about 15–20 min after sample mixing.

3. RESULTS AND DISCUSSION

Fig.1 shows the CD spectra in buffer solutions of natural (7*S*)-colchicine, unnatural (7*R*)-colchicine and each with tubulin (2:1) in the wavelength range between 250 and 400 nm. The CD spectrum of natural (7*S*)-colchicine is characterized by a major negative band with a maximum at about 340 nm and a minor negative band at about 265 nm (curve A), while that of unnatural (7*R*)-colchicine displays a mirror-image CD spectrum with positive CD bands (curve C). The optical characteristics exhibited by (7*S*)-colchicine and (7*R*)-colchicine appear to originate from the phenyl-tropolonic system (A and C rings), rather than the C-7 chiral center of ring B (scheme 1). Support for this conclusion is based on the following: In addition to atomic chirality at C-7, colchicine also possess axial chirality originating from the phenyl-tropolonic moiety. A recent NMR study [6] revealed that colchicinoids can undergo *aS-aR* phenyl-tropolonic isomerization [6,14] and the position of the *aS-aR* equilibria appears to be dependent upon the nature of the substituents at C-7 of ring B [6]. NMR spectral analysis indicated that both (C)-7 acetamido groups of natural (7*S*)- and unnatural (7*R*)-colchicine prefer the equatorial over the axial orientation. This conformational preference of the acetamido group causes natural (7*S*)-colchicine to adopt the *aS* configuration, whereas unnatural (7*R*)-colchicine adopts the *aR* configuration. Thus, the large negative rotation observed for natural (7*S*)-colchicine is attributed to the (*aS,7S*) conformer, whereas the positive rotation of unnatural (7*R*)-colchicine to the (*aR,7R*) conformer. Since

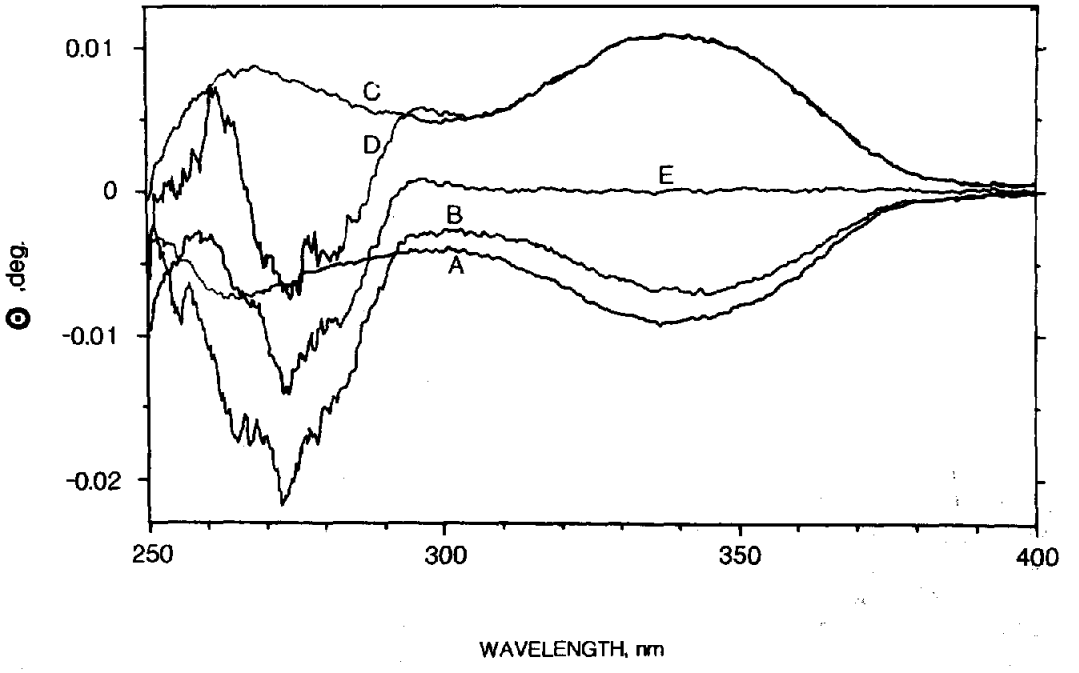
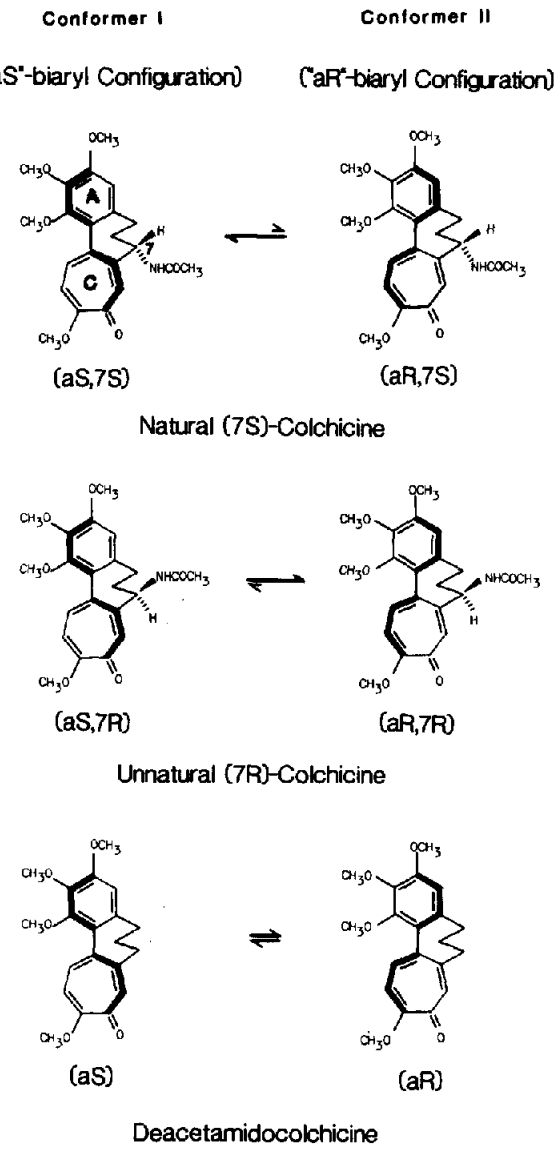


Fig.1. CD spectra of colchicine (40 μM) in the presence and absence of tubulin (20 μM) over the wavelength range 250–400 nm. Curves: (A) natural (-)-colchicine; (B) natural (-)-colchicine and tubulin (2:1); (C) unnatural (+)-colchicine; (D) unnatural (+)-colchicine and tubulin (2:1); (E) tubulin only.



Scheme 1.

(±)-deacetamidocolchicine (having only axial chirality) exhibited a net positive CD spectrum similar to that of unnatural (7*R*)-colchicine (having atomic and axial chiralities) in the presence of tubulin (as will be discussed later), the optical rotation exhibited by these colchicinoids therefore is attributed to the axial chirality originating from the phenyl-tropolonic moiety rather than the C-7 chiral center. This conclusion is further supported by optical measurements of a $CDCl_3$ solution of (7*S*)-1-acetyl-1-demethylcolchicine in which a reduction of negative rotation values ($[\alpha]_D$) from −185 at 22°C to −76 at 50°C [13] was accompanied, respectively, by a change in molar ratio of *aS* to *aR* conformers from about 1:0.4 to 1:0.8 as measured by NMR.

The CD spectrum of tubulin is characterized by a large negative band at about 272 nm and a small positive band at 295 nm (fig.1, curve E), presumably contributed by aromatic residues and disulfides of the protein. No CD absorption was observed above 310 nm for tubulin.

Addition of natural (−)-(7*S*)-colchicine to tubulin resulted in a notable reduction of the ellipticity above 310 nm (fig.1, curve B) when compared to an equal concentration of protein-free colchicine (curve A), whereas no discernible reduction was seen when unnatural (+)-(7*R*)-colchicine was added to tubulin (fig.1, curves C,D). These observations indicated that tubulin interacts with the former but not the latter enantiomer, and only with colchicine in the *aS*-biaryl configuration. The requirement for the *aS*-biaryl configuration of colchicine for binding to tubulin was demonstrated by a CD study of (±)-colchicine having equimolar amounts of (7*S*)- and (7*R*)-colchicine, and (±)-deacetamidocolchicine which possesses only axial chirality. Fig.2 shows the difference ellipticity at 340 nm when measured in the presence and absence of a fixed concentration of tubulin (20 μM) for (−)-(7*S*)-colchicine, (+)-(7*R*)-colchicine, (±)-colchicine and deacetamidocolchicine with drug enantiomer/tubulin ratios ranging from 0 to 3 mol/mol. The difference ellipticity ($\Delta\theta$, drug plus tubulin − drug only) at 340 nm when natural (-)-(7*S*)-colchicine is added to tubulin showed an increasing positive rotational strength which finally levelled off at molar ratios above ~ 1.25–1.5 (fig.2A), which is in reasonably good agreement with the 1:1 stoichiometry as reported from other binding studies [15]. Using the CD titration data together with a Scatchard plot, the association constant for the drug-tubulin (1:1) binding was roughly estimated at 0.1 μM^{-1}, which is within the range of reported values (0.05–5 μM^{-1}) [16,17]. No notable difference ellipticity was observed for unnatural (+)-(7*R*)-colchicine (fig.2B), confirming its lack of binding to tubulin. The titration of tubulin with (±)-colchicine (fig.2C), which showed a zero rotation value in the absence of tubulin

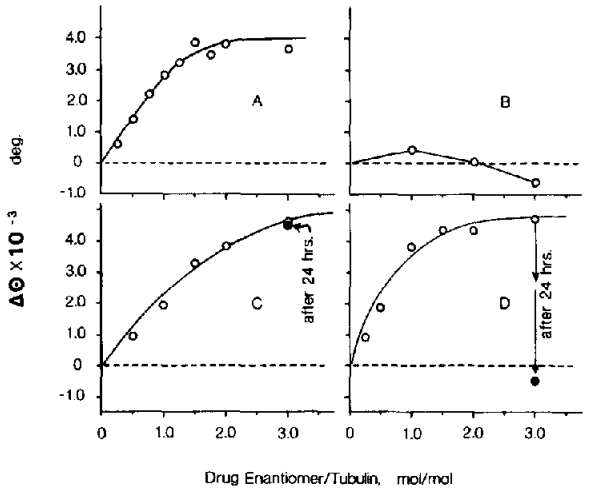


Fig.2. Difference ellipticities ($\Delta\Theta$) at 340 nm (drug plus protein - drug only) measured in the presence and absence of tubulin (20 μM) for natural (-)-colchicine (A), unnatural (+)-colchicine (B), racemic (±)-colchicine (C) and deacetamidocolchicine (D) at drug enantiomer/tubulin ratios in the range 0-3 mol/mol (enantiomer concentration is 0.5 of racemic mixture).

(not shown), afforded difference ellipticity at 340 nm with positive rotation values which increased and slowly levelled off. Such changes of CD spectra are expected since the negative CD band at 340 nm contributed by (-)-(7*S*)-colchicine is reduced upon binding to tubulin and leaves the unbound colchicine in solution with excess (+)-(7*R*)-colchicine, resulting in a net positive CD band. This net positive CD band remains unchanged even after 24 h, since unbound (7*R*)-colchicine remains predominantly in an *aR*-biaryl conformation regardless of binding of (7*S*)-colchicine to tubulin. However, this situation is significantly different for deacetamidocolchicine. Deacetamidocolchicine which has equal concentrations of *aS* and *aR* conformers also exhibits zero ellipticity at 340 nm in the absence of tubulin. Titration of tubulin with deacetamidocolchicine gave CD spectra similar to those observed with (±)-colchicine (fig.2D), confirming that it is indeed the *aS* conformer of deacetamidocolchicine which binds to tubulin, leaving the *aR* conformer unbound, and thus resulting in a positive CD band. Unlike (±)-colchicine, the net positive CD band of the unbound deacetamidocolchicine observed in the presence of tubulin was gradually reduced and finally disappeared after about 24 h. This agreed with our expectations since reestablishment of an *aS*-*aR* equilibrium from a predominantly *aR* conformer to equal concentrations of *aS* and *aR* conformers (which give no CD band), takes place at the rate of the biaryl isomerization, which has been reported to be in the range of 1×10^{-4} s^{-1} for a number of colchicinoids [6,14]. Although reduction of optical rotation of natural (-)-colchicine on binding to tubulin was previously reported [17], the present study with (±)-colchicine and (±)-deacetamidocolchicine provides the first demonstration that it is the (-)-(*aS*) enantiomer which binds to the protein, leaving the (+)-(*aR*) enantiomer unbound.

4. CONCLUSIONS

The present results provide evidence supporting the hypothesis that, in addition to the structural and spatial requirements (i.e. the positioning of the four methoxy groups in colchicinoids), it is the conformation of the phenyl-tropolone system in an *aS* configuration which is required for binding to tubulin. The CD data also demonstrate that the high negative rotations measured for colchicinoids stem from the *aS* configuration of the biaryl system rather than the contribution by the C(7) chiral center. Natural colchicine which binds to tubulin, therefore, should be referred to as (-)-(*aS*,7*S*)-colchicine.

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