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Stereochemistry of Colchicinoids. Enantiomeric Stability and Binding to Tubulin of Desacetamidocolchicine and Desacetamidoisocolchicine

ULF BERG*,[1] JOHANNA DEINUM,† PER LINCOLN,† AND JAN KVASSMAN‡

*Divisions of Organic Chemistry 3 and ‡Biochemistry, Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden, and †Department of Medical Physics, University of Göteborg, P.O. Box 33031, S-400 33 Göteborg, Sweden

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Desacetamidocolchicine (**3**) and desacetamidoisocolchicine (**4**) have been chromatographically resolved into enantiomers. Thermal racemization gives inversion barriers of 22.1 and 23.4 kcal mol^{-1}, respectively, for rotation around the bond joining the two aromatic rings. Kinetic binding experiments show that the enantiomer of **3** with the same helicity as native colchicine binds approximately 62 times as fast as colchicine to tubulin whereas the other enantiomer and both enantiomers of **4** do not bind. With molecular mechanics computations both the structures and the rotational barriers are reproduced, provided that the MM2(1985) force field, which gives stiffer aromatic rings than earlier force fields, is used.

INTRODUCTION

Colchicine, **1**, is the antimitotic constituent of meadow saffron (*Colchicum autumnale*). Numerous of its derivatives have been extensively investigated with respect to their binding to tubulin and concomitant biological activity in order to bring about an understanding of the effects of the drug on a molecular level (*1*). The stoichiometric binding of colchicine to isolated tubulin is a slow, strongly temperature-dependent, and poorly reversible process, which requires an activation energy of about 20 kcal mol^{-1}. Moreover, substoichiometric amounts of colchicine with respect to tubulin inhibit microtubule assembly (*2*). The kinetics of the binding to tubulin suggest a two-step process: a fast preequilibrium step followed by a slow, nearly irreversible step reflecting a ligand-induced conformational change in the protein (*3*).

$$T + C \overset{\text{fast}}{\rightleftharpoons} TC^* \overset{\text{slow}}{\rightarrow} TC \qquad [1]$$

Structurally, colchicine is characterized by the three rings of the molecule, the A ring or the trimethoxybenzene moiety, the seven-membered B-ring, and the methoxytropone or the C-ring. The A- and C-rings are indispensible for high

[1] To whom correspondence should be addressed.

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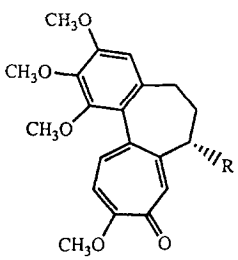
affinity binding although both trimethoxybenzene and methoxytropone bind independently to the active site with low affinity (*4*). S ev̈ studies show that colchicine derivatives with a modified B-ring are activ ..hthat both the binding rate and the stability of the complex are affected. Specifically, the analogue lacking the B-ring binds rapidly and reversibly to tubulin (*5*).

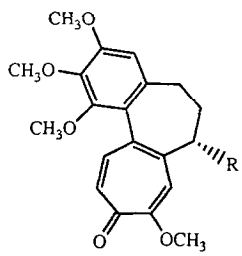
This report treats the conformational characteristics of the colchicine skeleton and its importance for the binding to tubulin. According to X-ray data colchicine and derivatives with intact ring systems have A- and C-rings twisted with respect to each other by 52 to 57° (*6*). Interestingly, the A–C compound **5** has an angle of 57° in the crystal, nearly identical with those of **1** and **2** (*7*). However, a new active analogue with a modified B-ring has an angle of 30° (*7b*).

On the basis of the effect on the CD spectrum of colchicine upon binding to tubulin Detrich *et al.* proposed that a conformational change of colchicine accompanies its binding to tubulin involving a boat–boat inversion of the tropolonic C-ring with concomitant decrease of the A–C dihedral angle from 53 to ≤19° (*8*).

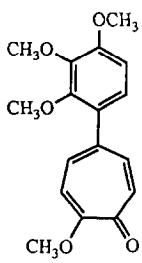
The mutarotation observed for isocolchicine (**2**) has been shown to result from rotation about the pivot bond joining the A- and C-rings, associated with a barrier of 23.7 kcal mol^{-1} (*9*). However, mutarotation has hitherto not yet been reported for colchicine **1.** Due to the presence of the acetamido function the atropisomers (ΔG° = 1.0–1.3 kcal mol^{-1} in **2**) are diastereomeric (*10*). Elimination of the acetamido group from **1** or **2** gives desacetamidocolchicine (**3**) and its isocolchicine analogue (**4**), which are instead enantiomeric with respect to rotation about the pivot bond. Racemization is in this case equivalent to a change in helicity. We now report on the optical resolution and rates of racemization of **3** and **4,** as well



R = $NHCOCH_3$ **1**
= H **3**



R = $NHCOCH_3$ **2**
= H **4**



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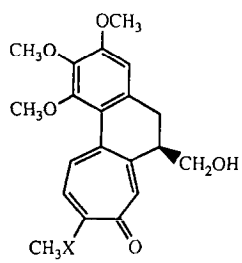

6 (X = O, S)

as the binding rates of their enantiomers to tubulin. The conformations have been characterized further by molecular mechanics computations.

EXPERIMENTAL

Materials. The two isomers were prepared from colchicine (purchased from Fluka) according to the classical sequence of Eschenmoser (*11*) and van Tamelen (*12*), separated by preparative HPLC on a Nucleosile straight-phase column and eluted by acetone. The compounds **3** and **4** had melting points (*11*), ir (*11*), uv (*11*), and NMR spectra (*13*) in agreement with the literature data, and were pure according to HPLC analysis.

Tubulin from microtubule proteins from bovine brain was prepared and characterized as described before (*14*).

Methods. Chromatographic resolution of enantiomers was performed by using a Conbrio-TAC column (*15*) packed with swollen, microcrystalline triacetylcellulose, with particle size 15–25 μm, 250 × 5 mm i.d. The instrumentation has been described earlier (*16*). The column was double-mantled and the temperature was regulated by circulating ethanol from a cryostat through the outer mantle. A solution of ca. 5 mg of racemic material in 1 ml ethanol was injected and allowed to pass the column and the detector system (a Perkin–Elmer Model 241 MC polarimeter and a LKB Uvicord 2138 S uv cell) before it was collected at −70°C. The dead volume (29 ml) of the column was determined by 1,3,5-tri-*tert*-butylbenzene.

^{1}H NMR spectra were recorded with a Nicolet 360WB spectrometer. CD spectra were recorded on a JASCO Model J41-A spectrometer, and uv spectra on a Cary 119 spectrometer. The cell for variable temperature CD measurements is described elsewhere (*17*).

Thermodynamic activation parameters were calculated from least-square Eyring plots.

The kinetics of the binding of colchicine derivatives to tubulin were measured by the stopped-flow fluorescence technique using the equipment described before (*18*) with an excitation wavelength of 365 nm and a Corning C.S. 3-72 cutoff filter in the emission path. The data were analyzed by a visual fit to

$$F_\infty - F_t = A_1 e^{-r_1 t} + A_2 e^{-r_2 t}, \qquad [2]$$

which describes the approach of the fluorescence F at time t toward the final fluorescence level F_∞ in terms of two exponential transients with amplitudes A_1 and A_2 and time constants r_1 and r_2. The error in the rate constants is of the order of 10% based upon estimated maximum deviations between experimental and calculated curves. Protein solutions were 0.1 M in Pipes, 0.5 mM in $MgCl_2$, 0.1 mM in GTP, and pH 6.8. The temperature was 21.0 ± 0.3°C in all experiments. Pseudo-first-order conditions were used with an excess of tubulin in order to avoid interference from small amounts of racemized substrate.

Molecular mechanics calculations were performed with the MM2/MMP2 programs developed by Allinger and co-workers (*19–22*), using both the 1977 and the

TABLE 1

Chromatographic Parameters for the Resolution of **3** and **4** on Swollen, Microcrystalline Triacetylcellulose at 5°C in 95% Ethanol[a]

	3			**4**		
Configuration	*P*		*M*	*M*		*P*
Capacity factor, k'	0.65		1.52	0.69		2.62
Relative retention, α		2.33			3.80	
Resolution, R_s		1.00			0.95	

[a] Flow rate, 0.5 ml/min.

1985 force fields for comparison. The construction of input structures was performed with the molecular modeling program MIMIC (*23*). Potential energy curves were calculated using the MM2 driver with full energy minimization, except for the dihedral angle used as the driving angle.

RESULTS

Enantiomer resolution, CD spectroscopy, and racemization. Optical resolution of **3** and **4** was performed by medium-pressure liquid chromatography on swollen, microcrystalline triacetylcellulose at 5°C giving essentially baseline separation and quantitative yield. The chromatographic parameters are given in Table 1. The enantiomer solutions were collected at −70°C and were enantiomerically pure according to analytical HPLC and tubulin binding experiments (see below). No racemization could be detected after 1 day at −20°C. The CD spectra were recorded immediately after resolution and the spectra of the first eluted enantiomers are shown in Fig. 1. The other enantiomers gave rise to the expected inversed spectra. The spectra are very similar, and also similar to those of colchicine and isocolchicine with only minor differences in peak position or intensity, but with one important exception: all the bands of the first eluted enantiomer of **3** have the same sign as those of native **1** (*24*), whereas those of **4** have opposite sign. Since native colchicine and isocolchicine derived from it have the same sign of their CD bands and P^2 configuration of their pseudobiaryl moieties, we may conclude that the first eluted enantiomer of **3** has the same configuration *P* as native colchicine, and that of **4** has opposite configuration *M*. The racemizations of **3** and **4** in ethanol were followed by monitoring the decline of the CD intensity of the strong bands around 232 nm over two to four half-lives in a thermostated cell at five temperatures in the range 15 to 60°C. First-order kinetics was observed. The rate constants for rotation around the pivot bond (k_{rot}) were calculated as $k_{rot} = \frac{1}{2}k_{rac}$, giving the following activation parameters for the rotation: compound **3**, $\Delta G^{\ddagger}_{300\,K}$

[2] *M*/*P* nomenclature (*25*) unequivocally defines the helicity of chiral atropisomers, and is independent of substituents in ortho positions in contrast to the *R*/*S* nomenclature.

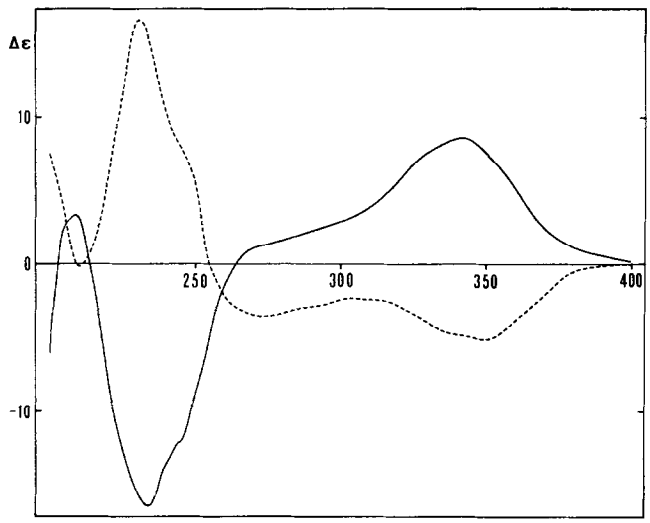

FIG. 1. Circular dichroism spectra of the first eluted enantiomer of desacetamidocolchicine (**3**; ---) and desacetamidoisocolchicine (**4**; —) in ethanol.

= 22.1 ± 0.2 kcal mol^{-1}, ΔH^{*} = 17.1 ± 0.3 kcal mol^{-1}, and ΔS^{*} = −16.4 ± 1.1 cal mol^{-1} K^{-1}; and compound **4**, ΔG^{*} 300 K = 23.4 ± 0.2 kcal mol^{-1}, ΔH^{*} = 19.3 ± 0.3 kcal mol^{-1}, and $\Delta S^{\neq}$ = −13.7 ± 2.1 cal mol^{-1} K^{-1}. The higher barrier of **4** than of **3** reflects the difference of the two valence tautomeric forms of the tropolone unit in accommodating the extra strain in the transition state. A comparison of **2** and **4** indicates that the acetamido function has only a minor effect on the barrier height, and we may extrapolate a value for the rotational barrier of **1** of 22.0–22.5 kcal mol^{-1}.

In an attempt to estimate the energy difference between the atropisomers of **1**, a sample of ca. 12 mg of the compound was dissolved in 1.2 ml of methanol-d_4, sealed into a glass ampoule and heated at 160°C for 4 h. The solution was quenched at −70°C and transferred to an NMR tube in the cold, and the ^{1}H NMR spectrum run at −60°C. The spectrum was identical with that of original **1**, indicating that $\Delta G^{\circ} \geq 2.2$ kcal mol^{-1} for the diastereomeric equilibrium in methanol.

A further experiment that may give some information about the conformational flexibility of colchicine derivatives is obtained from the temperature dependence of the CD spectra (*17*). The existence of conformational equilibria can be observed by CD spectroscopy even when the barrier separating the conformers is much too low to permit the observation of individual conformers by low-temperature NMR spectroscopy. The temperature dependence of the CD spectra of colchicine and desacetamidoisocolchicine was studied in ethanol between room temperature and −100°C (Fig. 2). Desacetamidoisocolchicine shows a negligible dependence which is within the limit of experimental error. Colchicine likewise has a very small temperature dependence with the only exception being the moderate increase of the band at 224 nm. This band lies in the region for the amide $\pi \rightarrow$

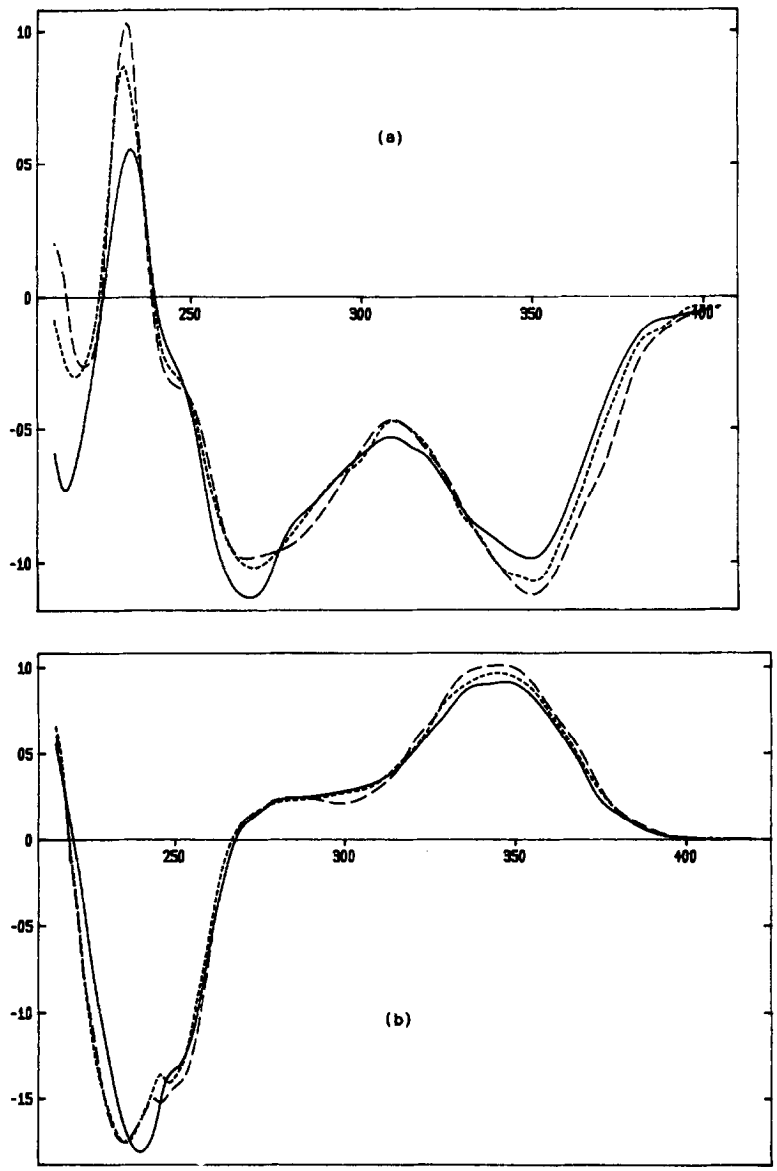

FIG. 2. Temperature dependence of CD spectra of (a) colchicine (**1**) and (b) desacetamidoisocolchicine (**4**): in ethanol; (—) 24°C; (---) −39°C; (–––) −95°C.

π^* transition, and may indicate an equilibrium between different conformations of the acetamido group.

Kinetics of the binding to tubulin. The accessibility of enantiomerically pure (+)- and (−)-desacetamidocolchicine (**3**) and (+)- and (−)-desacetamidoisocolchicine (**4**) offers a possibility to study the rate of binding to tubulin of each enantio-

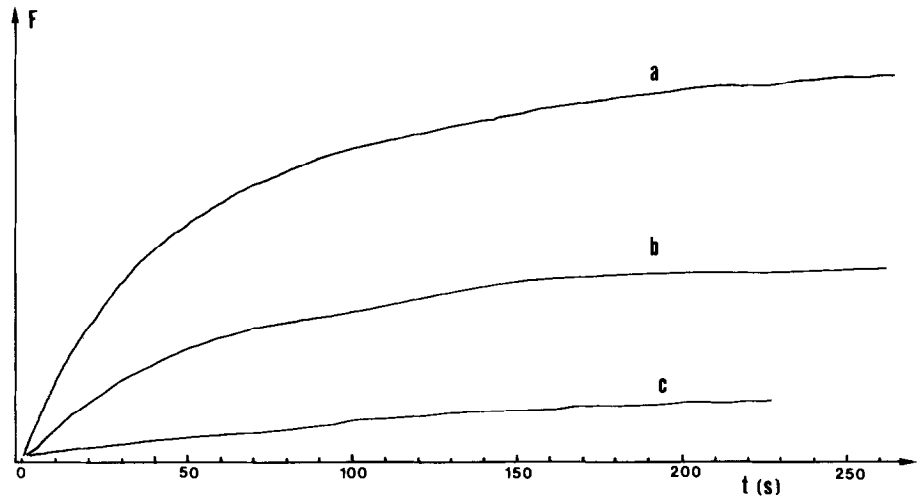

FIG. 3. Stopped-flow fluorescence development for the binding to tubulin at 21°C of (−)-**3** (trace a), (+)-**3** (trace c), and (+)-**3** after racemization (trace b). The wavelength of excitation was 365 nm and of emission 465 nm. Fluorescence in arbitrary units.

mer differing only in their helicity around the pivot bond. Binding of racemic desacetamidocolchicine, **3**, to tubulin has been verified by competition experiments with [^{3}H]colchicine (*32*). The kinetics of the association were followed by the stopped-flow technique under pseudo-first-order conditions with an excess of tubulin (initial concentrations, 13.5 μM in tubulin and 2 μM in drug). The curves of fluorescence versus time for (−)-**3**, (+)-**3**, and for (+)-**3** after racemization at 45°C are shown in Fig. 3. Under these conditions neither (+)-**4** nor (−)-**4** showed any significant fluorescence. In control experiments native colchicine was studied under pseudo-first-order conditions with excess tubulin as well as with excess colchicine and gave identical results within the experimental error. The results are shown in Table 2 in which the rate constants include the preequilibrium step in

TABLE 2

Second-Order Rate Constants for the Binding of Colchicine and Both Enantiomers of **3** and **4** to Tubulin Evaluated from the Development in Fluorescence[a]

Compound	k (M^{-1} s^{-1})	
	Fast phase	Slow phase
(−)-Colchicine	48	6.7
(−)-**3**	3.8×10^3	8.5×10^2
(+)-**3**	(2×10^{-3}; see text)	—
(+)-**3** after racemization	3.6×10^3	11.2×10^2
(−)-**4**	No fluorescence	
(+)-**4**	No fluorescence	

[a] Temperature, 21°C.

Eq. [1]. The importance of the helicity alone is clearly shown by the 10^6-fold difference in binding rate between the enantiomers of **3.** The slow binding rate of (+)-**3** is of the same order of magnitude as the rate of racemization, suggesting that this enantiomer does not bind. After racemization (+)-**3** binds with essentially the same rate as the pure (−)-isomer but only to the extent that this isomer contributes to the racemic mixture as seen by the approximately half amplitude of the fluorescence (Fig. 3). This is readily understood by the 37-fold larger first-order rate constant for binding than for racemization at 21°C. Neither enantiomer of desacetamidoisocolchicine, **4** shows an increase in fluorescense in the presence of tubulin. It might be of some interest to note that the (+)-isomer of **4** has the same spacial relation between the methoxycarbonyl moiety of the tropolone ring and the helicity as the active enantiomer of **3,** but in (+)-**4** the side of attachment of the 1- and 2-methoxy groups in the A-ring as well as the side of annelation of the B-ring are reversed compared to the active forms of **1** and **3.** The absence of an enhanced fluorescence with **4** is consistent with lack of binding of isocolchicines shown before (*26*).

Molecular mechanics computations. In order to gain some further insight in the structural rigidity of the colchicine system we decided to try some empirical force-field calculations. A conformational analysis of colchicine and isocolchicine by the molecular mechanics MMX program, suitable for nonplanar conjugated double bonds, has been reported (*27*). In that report the general features of the experimental X-ray structures were reproduced by the calculations, but a few important discrepancies emerged. First, the conformational energies of the atropisomers were badly reproduced, which may be a result of different solvation. Second, and more important, the amply discussed puckering of the C-ring was calculated to be very different from the values in the various X-ray structures of both colchicine and isocolchicine derivatives. This is most easily seen by comparing the sum of the absolute values for the dihedral angles in the tropolone ring, $\Sigma|\phi|$. These values are 13, 47, 28, and 38°, in the four different forms in the crystals of isocolchicine and colchicine but were calculated as 144, 141, 30, and 73°, respectively, in the corresponding conformations with the MMX force field (*27*). We chose, instead, to use two versions of the MM2/MMP2 program, which in this context differ mainly in their way of generating torsional force constants for conjugated systems (*21, 22*). The older version, MMP2(1977), which is known to produce much too soft π-systems, gives torsional angles in the tropolone ring essentially concurring with those of the MMX force field, whereas MM2(1985), which has proven to give more realistic structures for strained aromatic systems calculates $\Sigma|\phi|$ values of 19 and 8°, respectively, for **3** and **4,** much more in agreement with experiment. The magnitude of this discrepancy is clearly seen from the relative position of the carbonyl oxygen atom as calculated by the two force fields, which differ by 2.0 Å in the two calculated structures![3] Therefore, we

[3] Tropone and several of its derivatives are planar in the gas phase and the vibrational spectrum of tropone in the gas phase confirms the inherent softness of the ring (*36*). These structural features are well reproduced by calculations with the MM2(1985) force field but not by the MM2(1977) or MMX force field.

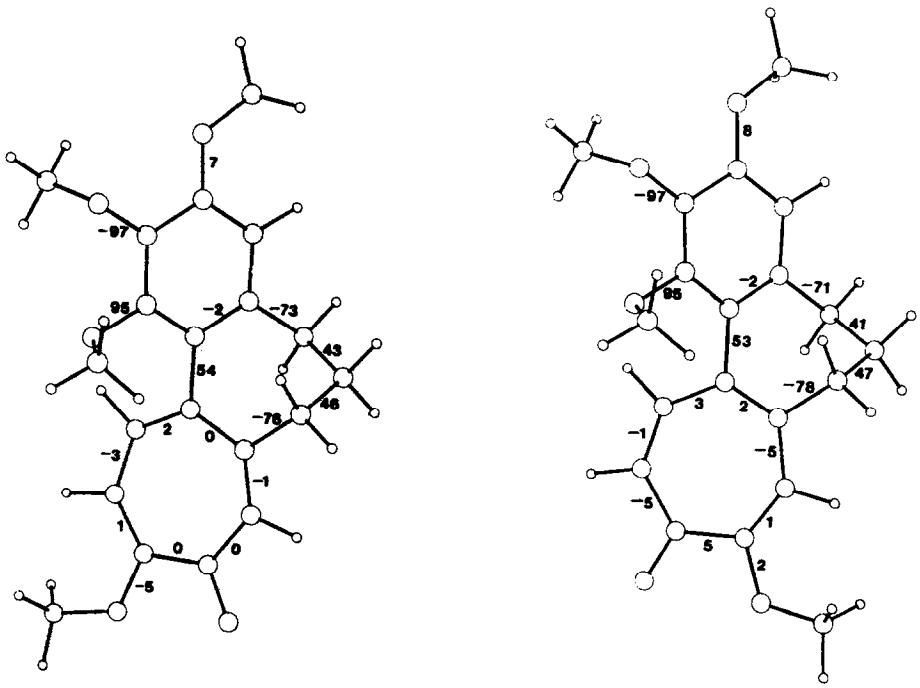

FIG. 4. Calculated structures of the low energy conformations of the *M* enantiomers of **3** and **4** including selected dihedral angles.

consider all proposals on conformational changes of the C-ring based on calculations or model inspections as highly speculative. Suffice it to state that the tropolone ring is rather soft due to the ring strain in the planar seven-membered ring, and thus is susceptible to minor departures from an essentially planar ground-state structure upon interactions with tubulin. A clear illustration of this flexibility is seen in the large difference of $\Sigma|\phi|$ between the two structures in the crystal of **2** (*28*). In the following discussion results from the MM2(1985) force field are presented.

Calculated structures for **3** and **4** are shown in Fig. 4 including some selected dihedral angle values. The low-energy conformer of **3** was calculated to be 0.80 kcal mol^{-1} more stable than that of isomer **4.** We next address the barrier to rotation around the pivot bond. The ground-state value for the dihedral angle between the two aromatic planes was calculated as 53.6° for **3** and 52.7° for **4** in good agreement with the X-ray structures (*28, 29*). The potential energy curve for rotation through the planar state for **3** is shown in Fig. 5 together with the one for the A–C compound **5.** The rotational barrier in **3** was calculated as 20.8 kcal mol^{-1}, and no local minimum other than the global one was found with respect to rotation around this bond. In **5** the situation is slightly different. There are two conformations corresponding to colchicine and isocolchicine structures of the A–C moieties differing by only 0.02 kcal mol^{-1}, and separated by a barrier of only

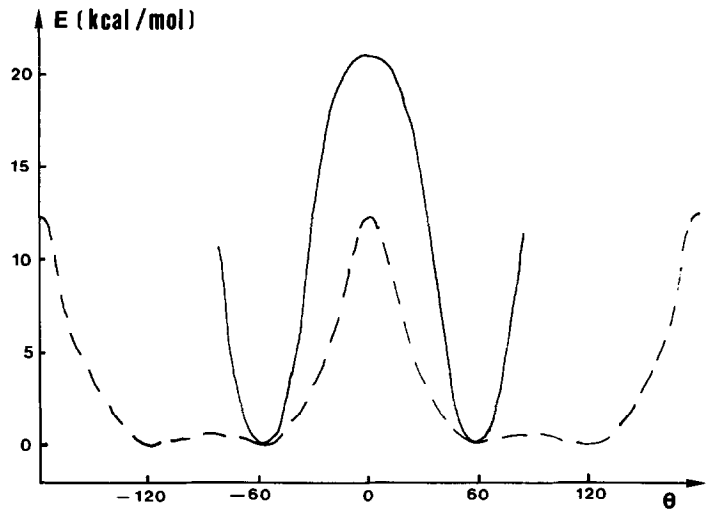

FIG. 5. Potential energy versus dihedral angle between A- and C-rings for **3** and **5** as calculated by the MM2(1985) force field: **3** (—); **5** (---).

0.4 kcal mol^{-1}. In the crystal the isocolchicinoid conformation is found (*7*). The ground-state dihedral angles are calculated as 54.6 and 56.6° which should be compared to the experimental value 57.4°. The barrier to rotation through the planar form is predicted to be 12.2 kcal mol^{-1}, substantially lower than that of **3**, but still far from a barrier corresponding to free rotation as it is usually characterized in **5**.

Finally, the conformations of the methoxy groups were considered in **3**. The most stable conformation has the 1- and 2-methoxy groups perpendicular to the aromatic ring and antiparallel to each other such that the methyl in the 1-substituent points away from the hydrogen on the 12-carbon in the tropolone ring. The parallel conformation, which is found in some of the structures in the crystal (*28, 29*), is calculated to be a local minimum 1.3 kcal mol^{-1} higher in energy. The 3-methoxy group is more flexible, is essentially coplanar with the ring in the most stable conformation, and has a rotational barrier of 2.5 kcal mol^{-1}.

DISCUSSION

The results presented here offer a basis for a better understanding of the stereochemical features of colchicine and its derivatives. We found unambiguously that only (−)-**3** could bind to tubulin, while(+)-**3** did not bind at all. First, the importance of the helicity around the pivot bond for tubulin binding was thus clearly demonstrated and is in agreement with the finding by Yeh *et al.* that binding of racemic desactamidocolchicine results in a positive CD band from unbound (+)-**3** (*30*). Furthermore, certain proposals concerning major conformational changes of colchicine upon binding to tubulin have to be reconsidered. Thus it has been postulated that the second step of the binding of colchicine to tubulin is accompa-

nied by conformational changes about the pivot bond leading to planar or near planar conformers (*8, 31, 32*). Our finding of an increase in energy by 22–23 kcal mol^{-1} going from a twist of ca. 53° in the ground state to coplanar A- and C-rings seems incompatible with this hypothesis. Moreover, a conformation with a twist angle of 19°, which has been suggested as a stable and possible active form (*8*) is not a stable conformation and has an energy of 15–18 kcal mol^{-1} above the ground state according to molecular mechanics computations (Fig. 5).

Comparatively rigid ground-state structures with torsional angles about the pivot bond of ca. 53°, and essentially planar A- and C-rings were calculated for both **3** and **4.** Similar conclusions have recently been presented based upon resonance Raman spectroscopy (*33*), uv spectroscopy (*34*), and molecular mechanics calculations (*27*). Although we rule out the possibility of planar or even 19° twisted conformations in the colchicine–tubulin complex, there is still a theoretical possibility that such conformations are present as transient intermediates in the binding process. As a matter of fact the activation energies for binding of colchicine and the A–C analogue **5** match the rotational barriers quite well, being 20–24 and 13 kcal mol^{-1}, respectively. Even though there is no evidence bearing on this issue we favor an interpretation in which the major part of the activation of binding lies on changes in the protein conformation. A support for a change in the protein conformation has been found in the different reactivities of the sulfhydryl groups of tubulin in the presence of colchicine (*14*). The very different reaction and activation entropies for binding seem to suggest a complicated process and are difficult to rationalize by differing properties of the drugs.

The origin of the circular dichroism displayed by the colchicine chromophore is not fully understood. It is obvious that the broad band centered around 340 nm is composed of more than one transition, maybe more than two. A rigorous analysis of the CD spectrum of colchicinoids is thus impossible at present, as is an explanation to the interesting observation that the CD band of colchicine around 340 nm disappears upon binding to tubulin. An indication that the vanishing CD band is not due to more planar conformations around the A–C bond is found with thiocolchicine, which exhibits reversed sign of one of its CD bands around 340 nm on binding (*35*). Alternative explanations in terms of conformational changes of the tropolone ring or ring stacking interactions with tryptophan residues at the binding site have been proposed. The observation that the analogue with a six-membered B-ring, **6** (X = S), binds to tubulin despite a much smaller twist angle between the A- and C-rings of 30° (*7b, 35*), however, shows that an angle around 53° is not a requirement for binding.

In conclusion, the stereodynamic properties of colchicine and its derivatives reported here give a better basis for the interpretation and understanding of the binding to tubulin of these drugs and of their biological effects in general.

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