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Articles

Conformation of Thiocolchicine and Two B-Ring-Modified Analogues Bound to Tubulin Studied with Optical Spectroscopy

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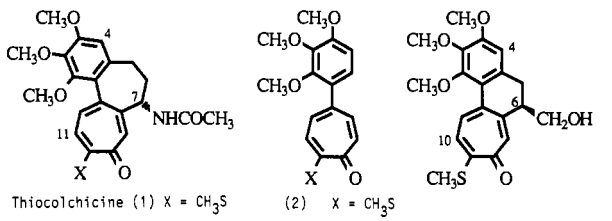
ABSTRACT: The interaction of tubulin with thiocolchicine and two thiocolchicine analogues, one lacking the B ring and one with a six-membered B ring, has been studied by using near-UV and CD spectroscopies. Rapid, reversible binding of the latter analogue to tubulin demonstrates the ability of the colchicine binding site to accommodate the phenyltropone system with a more coplanar conformation than is present in free colchicine. There is no evidence, however, that bound thiocolchicine should have a much less twisted conformation than free thiocolchicine. Thiocolchicine and the bicyclic analogue appear to have approximately the same conformation of the phenyltropone system, in both the free and the bound states, suggesting that this conformation has an optimal arrangement of the phenyl and tropone rings for binding to tubulin. In contrast to colchicine and related derivatives, the three thiocolchicine analogues show pronounced near-UV CD bands upon association to tubulin. No simple relation could be found between the sign pattern of the CD components in the near-UV band of the thiocolchicinoid chromophore and its axial chirality.

Colchicine (Chart I), the classical mitosis-inhibiting alkaloid from *Colchicum autumnale*, mainly exerts its physiological activity by forming a stable 1:1 complex with the tubulin protein, thereby interfering with its assembly to microtubuli (Bryan, 1972; Olmsted & Borisy, 1973; Sternlicht & Ringel, 1979; Farell & Wilson, 1984). Colchicine is not covalently bound to tubulin, but the high activation energy for binding and dissociation makes the binding slow and practically irreversible (Garland, 1978; Lambeir & Engelborghs, 1981). Studies of more simple analogues of colchicine have shown that the trimethoxybenzene and methoxytropone rings each bind to distinct sites (Cortese et al., 1977; Andreu & Timasheff, 1982a,b) and that the direct linkage of the rings, by virtue of a chelate effect, is a prerequisite for high binding affinity (Andreu et al., 1984). The B ring, including its substituents, has a major influence on the association/dissociation kinetics (Bhattacharyya et al., 1986; Banerjee et al., 1987). For example, the bicyclic colchicine analogue MTC[1]

Chart I



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[1] Abbreviations: CD, circular dichroism; DMSO, dimethylsulfoxide; LD, linear dichroism; LD′, reduced linear dichroism; MCD, magnetic circular dichroism; MTC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; Pipes, 1,4-piperazinediethanesulfonic acid; PVA, poly(vinyl alcohol).

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[2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one] of Fitzgerald (1976) exhibits rapid and reversible binding to tubulin (Bane et al., 1984; Engelborghs & Fitzgerald, 1986, 1987).

It has recently been shown that the *a*S configuration of the phenyltropone system is a necessary condition for tubulin binding and that it is the axial chirality of the molecule rather than the chiral center at C-7 that determines the chiroptical properties of the colchicines (Yeh et al., 1988). Nevertheless, neither colchicine (Detrich et al., 1981), desacetamidocolchicine (Yeh et al., 1988), nor MTC (Andreu et al., 1984) displays any significant CD in the low-energy absorption band of the phenyltropone chromophore (around 350 nm) when associated to tubulin.

The disappearance of the negative CD band at 340 nm in free colchicine upon binding to tubulin has been suggested to indicate a different conformation in the bound state (Detrich et al., 1981). The dihedral angle (ϑ) between the phenyl and tropone ring planes is 53° and 51° for two independent molecular configurations in the colchicine crystal (Lessinger & Margulis, 1978), and proton NMR simulations indicate a similar conformation in solution (Brossi et al., 1988; Donaldsson, 1988). A conformation with $\vartheta \sim 19°$, and thus "lower" axial dissymmetry, has been proposed for colchicine bound to tubulin (Detrich et al., 1981). The difference in binding activation energy between colchicine and MTC, as well as the increased fluorescence quantum yield of bound colchicine, has been taken as evidence supporting the hypothesis of a more planar conformation of tubulin-bound colchicinoids (Detrich et al., 1981; Bane et al., 1984; Bhattacharyya et al., 1986). The different tubulin-binding properties of 4- and 6-chloro-MTC have been interpreted in similar terms (Banwell et al., 1988). However, a colchicine conformation with such a small dihedral angle has recently been questioned for energetic reasons, on the basis of molecular mechanics calculations (Donaldsson, 1988).

Thiocolchicine derivatives, where the oxygen in the tropone methoxy group has been replaced by sulfur, are generally equally good or better competitors for [^{3}H]colchicine binding to tubulin compared to the oxo analogues (Kerekes et al., 1985; Dumont et al., 1987). The rate constant for binding to tubulin at 37 °C is about four times larger for thiocolchicine (**1**) than for colchicine (Chabin & Hastie, 1989). The near-UV spectrum of **1** is perturbed by binding to tubulin, and resonance Raman spectroscopy indicates ring-stacking interactions between the tropone ring and an aromatic amino acid residue in the complex (Rava et al., 1987). Finally, 3-*O*-demethylthiocolchicine has been put forward as a promising broad-spectrum cytostaticum with markedly lower toxicity than colchicine (Kerekes et al., 1985; Brossi et al., 1988).

In the present study we observe that **1**, upon binding to tubulin, displays a pronounced CD at the position of the low-energy absorption band. With the aim of further exploring the tubulin interactions of the thiocolchicine chromophore, compound **2**, the thio analogue of MTC, was synthesized. Interestingly this compound, which is optically inactive, was found to exhibit a strong induced CD upon binding to tubulin. This observation motivated study of another analogue, with a more coplanar phenyltropone chromophore, in order to better understand how the spectral properties depend on the conformation. By use of a ring contraction described in the colchinol series (Cohen et al., 1940; Cook et al., 1952), the deamination of *N*-desacetylthiocolchicine with nitrous acid gave a complex mixture of products, from which **3** was isolated in low yield. This novel compound, with a six-membered B ring, was also found to bind rapidly and reversibly to tubulin. The UV absorption and circular dichroism spectra of the three thiocolchicine analogues, free and associated to tubulin, were studied in an attempt to assign their conformations in the bound state.

Materials and Methods

Protein Preparation. Microtubule protein was prepared from bovine brain in the absence of glycerol by three temperature-controlled cycles of assembly/disassembly in the presence of 1 mM GTP and 0.5 mM $MgSO_4$ (Deinum et al., 1984). Tubulin was separated from the microtubule-associated proteins by ion-exchange chromatography on Mg(II)-treated phosphocellulose (WhatmanP11) in 20 mM Pipes/0.5 mM $MgSO_4$ at pH 6.8 (Williams & Detrich, 1979), followed by gel filtration on Sephadex G-25 Fine (Pharmacia) in 0.1 M Pipes/0.5 mM $MgSO_4$. When required, tubulin was concentrated by pressure dialysis with an Amicon ultrafiltration cell at 4 °C. All protein solutions were clarified by ultracentrifugation and were stored in liquid nitrogen after drop-freezing.

The concentration of tubulin was determined by using ϵ(278 nm) = 0.134 μM^{-1} cm^{-1} (Deinum et al., 1981b). The microtubule protein concentration was determined with Bio-Rad's protein assay, with tubulin as the protein standard. The thiocolchicine analogues and allocolchicine were dissolved in ethanol prior to addition to buffer or protein solutions. In no case did the final concentration of ethanol exceed 3% (v/v).

Microtubule Assembly. Assembly was measured in 100 mM Pipes buffer, 0.5 mM $MgSO_4$, and 1 mM GTP at pH 6.8. Concentrated ice-cold microtubule protein was added to a buffer (at 37 °C) containing varying concentrations of **3** to a final protein concentration of 3 g L^{-1}. The temperature in the cuvette was controlled by a water jacket. Assembly, initiated by raising the temperature to 37 °C, was monitored through the increase in turbidity, measured as apparent absorbance at 450 nm to avoid interference from absorption of the thiocolchicine derivative. The half-maximum inhibitory concentration, I_{50}, was defined as the drug concentration giving a polymerization plateau value, which was 50% of the control without drug.

Optical Spectra. Absorbance spectra and time-dependent absorbance were recorded in a temperature-controlled cell by using a Perkin-Elmer 571 or a Varian Cary 219 spectrophotometer. Circular dichroism spectra were recorded on a Jasco-J500 spectropolarimeter and fluorescence emission spectra (excitation at 380 nm) on an Aminco Spectrofluorometer (corrected spectra), again in a thermostated cell. MCD spectra were recorded on the Jasco instrument using a rotatable permanent magnet, designed and calibrated as previously described (Nordén et al., 1977).

NMR Spectra. ^{1}H NMR spectra were recorded at 270 MHz on a Bruker WH 270 spectrometer. Resolution of the aliphatic five-spin system in compound **3** was accomplished by computer fitting using the simulation program ITRCAL supplied by Bruker. Initial estimates of coupling constants and resonance positions were obtained by spin decoupling.

Dichroic Film Spectra. A 10% PVA [poly(vinyl alcohol)] solution was prepared as described elsewhere (Matsuoka & Nordén, 1982). The PVA solution was mixed with a suitable quantity of the sample, centrifuged, and poured onto a horizontal glass plate and was then allowed to dry in a dust-free place (12 h). The thickness of the dry film was approximately 0.10 mm. Dichroic absorption spectra were measured on a Cary 219 spectrophotometer equipped with a rotatable Glan polarizer (Bernard Halle, Berlin) in front of the sample in the

light path. Differentially measured linear dichroism (LD = $A_{\parallel} - A_{\perp}$) was recorded on the Jasco J 500 spectropolarimeter by using the quarter wave and calibration technique described elsewhere (Nordén & Seth, 1985). The absorbances $A_{\parallel}$ and $A_{\perp}$ of sample and reference films were measured with the light polarized parallel and perpendicular, respectively, to the stretch direction of the film. The reduced linear dichroism, LD^r, was calculated as $LD^r = 3(A_{\parallel} - A_{\perp})/(A_{\parallel} + 2A_{\perp})$ (Nordén, 1978).

Chemicals. Colchicine (90–95%) was obtained from Sigma and used without further purification. MTC was obtained as generous gifts of Prof. Thomas J. Fitzgerald, School of Pharmacy, Florida A & M University, Tallahassee, FL, and Prof. James C. Lee, St. Louis University School of Medicine, St. Louis, MO. Organic solvents were of spectroscopic grade, buffer constituents of analytical grade, and other chemicals of reagent grade. Allocolchicine was prepared as described by Fernholtz (1950).

Preparation of Thiocolchicine Analogues. Thiocolchicine (**1**) and *N*-desacetylthiocolchicine were prepared from colchicine according to Velluz and Muller (1954a,b), melting points 190 °C (lit. 192 °C, loc. cit.) and 192–194 °C (lit. 198 °C, loc. cit.), respectively.

2-(Methylthio)-5-(2',3',4'-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (*2*). MTC (38 mg, 0.13 mmol) and *p*-toluenesulfonic acid monohydrate (13 mg, 0.07 mmol), in a test tube equipped with a screwcap, were immersed in an acetone/dry-ice bath. Methanethiol (2 mL) was added, and the tube was capped and left at room temperature in the dark. After 6 days, the tube was cooled in ice–water and opened and the excess of methanethiol was allowed to evaporate at this temperature. The residue was taken up in dichloromethane (2 mL), washed once with saturated aqueous $NaHCO_3$ and twice with water, dried (Na_2SO_4), and evaporated to dryness in vacuo. The residue was flash chromatographed on a 1.5 × 30 cm silica gel column set up in dichloromethane. The column was eluted first with 200 mL of dichloromethane and thereafter with 300 mL of dichloromethane–ethyl acetate 6:1 to afford the desired product as a yellow oil after evaporation of the solvents. Recrystallization from hexane gave 12 mg (30%) of **2** as yellow crystals: mp 103–105 °C; ^{1}H NMR (270 MHz, $CDCl_3$; ppm rel TMS; *J* in Hz) δ 2.42 (3 H, s, SCH_3), 3.73 (3 H, s, OCH_3), 3.90, 3.92 (6 H, d, $2OCH_3$) 6.73 (1 H, d, *J* = 8.6; 5'-H), 6.97 (1 H, d, *J* = 8.6; 6'-H), 7.03 (1 H, d, *J* = 10.0; 3-H), 7.07 (1 H, d, *J* = 12.2, 7-H), 7.17 (1 H, dd, *J* = 1.7, 10.0; 4-H), 7.44 (1 H, dd, *J* = 1.7, 12.2; 6-H).

5,6-Dihydro-6-(hydroxymethyl)-1,2,3-trimethoxy-9-(methylthio)-8H-cyclohepta[a]naphthalen-8-one (*3*). A stirred mixture of toluene (4 mL to suppress foaming) and a solution of *N*-desacetylthiocolchicine (0.375 g, 1.0 mmol) in 20% aqueous acetic acid (2 mL) was treated during 30 min with solid $NaNO_2$ (0.105 g, 1.5 equiv) in portions at 0 °C. Stirring was continued for 2 h at this temperature, whereupon the mixture was neutralized with solid NH_4HCO_3, diluted with water (10 mL), and extracted with dichloromethane (3 × 15 mL). The combined organic phases were washed with water, dried ($MgSO_4$), and evaporated to dryness in vacuo. The residue was flash chromatographed on a 4 × 20 cm silica gel column set up in ethyl acetate. Elution with ethyl acetate (0.5 L) removed most of the less polar byproducts. Further elution with ethyl acetate–ethanol 6:1 (0.5 L) afforded **3** as yellow-orange crystals after evaporation of the solvents and crystallization from ethyl acetate: 62 mg (17%), mp 200 °C; ^{1}H NMR (270 MHz, $CDCl_3$; ppm rel TMS; *J* in Hz) δ 2.43 (3 H, s, SCH_3), 2.94 (2 H, m, *J* = 15.2, 4.4, 3.6; 5-H), 3.05 (1 H, m, *J* = 7.7, 6.2, 4.4, 3.6; 6-H), 3.41 (1 H, m, *J* = 11.5, 7.7; 12-H), 3.59 (1 H, m, *J* = 11.5, 6.2; 12-H), 3.67 (3 H, s, OCH_3), 3.90 (6 H, d, $2OCH_3$), 6.60 (1 H, s, 4-H), 7.04 (1 H, d, *J* = 10.2; 10-H), 7.18 (1 H, s, 7-H), 7.91 (1 H, d, *J* = 10.2; 11-H).

Results

Structures of 2 and 3. The ^{1}H NMR spectrum of **2** confirms the anticipated structure of this compound and shows that the substitution of the 2-methoxy group in MTC has occurred in the ipso position. The assignment of the resonances was made by inference from ^{1}H NMR data of colchicine and isocolchicine derivatives (Wildman & Pursey, 1968; Hufford et al., 1980). Note the weak four-bond coupling constant of 1.7 Hz between C-4 H and C-6 H, which may be compared with the four-bond coupling constant of 3.2 Hz reported for isocolchicide (Danieli et al., 1985).

The ^{1}H NMR spectrum of compound **3** is consistent with this compound being the thiocolchicine equivalent of "Carbinol A" of Cook et al. (1952). Resolution of the aliphatic five-spin system indicates that the C-6 proton couples by 4.4 and 3.6 Hz to the two C-5 protons. The two small gauche coupling constants indicate an equatorial position for C-6 H in the B ring and thus that the hydroxymethyl group is axially oriented. There was no indication of the presence of the other atropisomer of **3** in $CDCl_3$, DMSO-d_6 or D_2O/DMSO-d_6 (8:2) solutions. The axial orientation of the hydroxymethyl group is in contrast to the situation in colchicinoids with a seven-membered B ring, where C-7 substituents seem to prefer a pseudoequatorial orientation (Brossi et al., 1988). Dreiding models indeed support the idea that compound **3** (with planar A and C rings) displays an unfavorable steric interaction between an equatorially oriented hydroxymethyl group and the C-7 hydrogen of the tropone ring. The ^{1}H NMR spectrum of **3** in toluene-d_8/CD_2Cl_2 solution showed no significant changes of the aliphatic spin system upon lowering the temperature to −90 °C. An X-ray crystallographic study of **3** has confirmed the proposed structure, showing a single enantiomer in the crystal with a dihedral angle ϑ of 30° and an axially oriented C-6 hydroxymethyl group (Olsson et al., 1989).

Interactions with Tubulin. The new colchicinoid compound **3** was found to inhibit assembly of microtubuli proteins with an I_{50} of about 13 μM. Incubation of compounds **1**–**3** with a 2-fold stoichiometric excess of tubulin produced marked changes in both the absorption and circular dichroism spectra above 320 nm, where all intensity derives from the drug chromophore (Figure 1). Increasing the protein–ligand ratio did not further change the absorption or circular dichroism spectra of **1**–**3**, indicating negligible amounts of free ligand. No spectral change was observed for any of the compounds, if the tubulin had first been incubated with an excess (2:1) of allocolchicine for 20 min at 37 °C. If the allocolchicine was added after the incubation of **1**–**3** with tubulin, the spectral changes were completely reversed within 20 min for **2** and **3**, indicating complete dissociation, while no significant reversal was noticed with **1**. Allocolchicine is a potent competitor to colchicine for binding to tubulin, and the fact that it has no absorption above 340 nm makes it a sensitive control for colchicine-type interactions with tubulin (Deinum et al., 1981a; Medrano et al., 1989; Hastie, 1989). We may thus conclude that the spectral effects of the interaction of tubulin with compounds **1**–**3** are indeed due to binding to the colchicine site of tubulin.

The binding of **2** and **3** to tubulin is fast and, as indicated by the spectral changes, reaches completion within the time of mixing with protein (ca. 30 s), even at +4 °C. By contrast, compound **1** was found to bind very slowly at 4 °C, while at

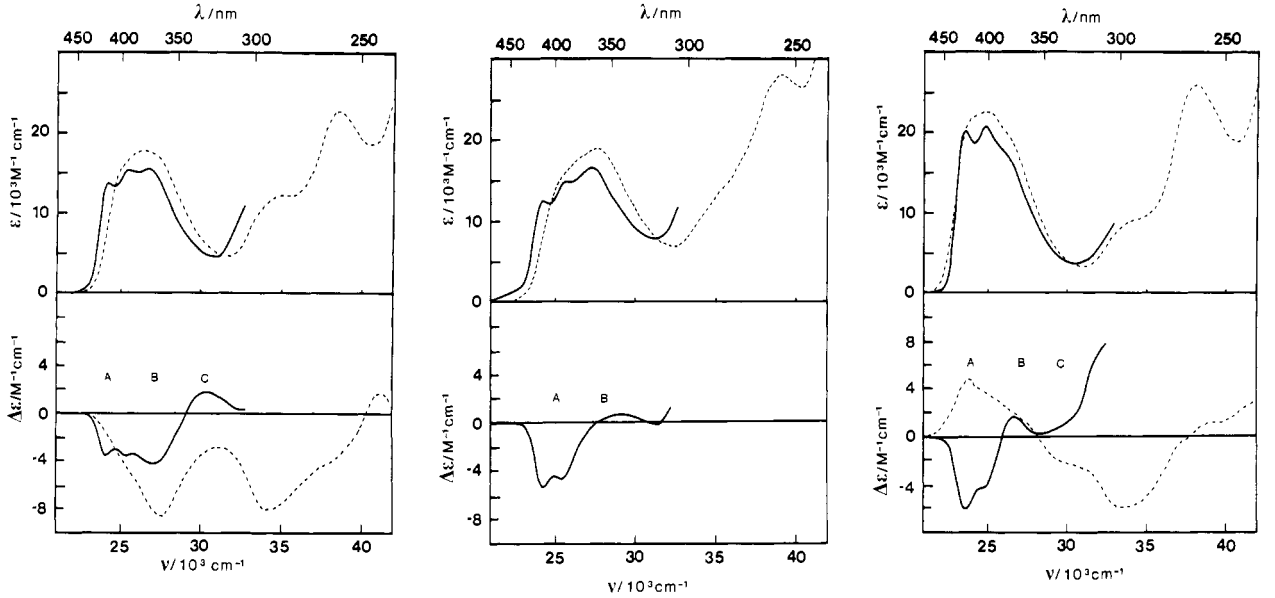

FIGURE 1: Absorption and circular dichroism spectra of thiocolchicine and analogues after incubation at 37 °C with excess tubulin in buffer, superimposed on spectra in buffer. Absorbance recalculated to molar units. (a, Left) Absorption and circular dichroism spectra of **1** (46 μM) in buffer containing 90 μM tubulin (solid curve) and in pure buffer (broken curve). (b, Middle) Absorption and circular dichroism spectra of **2** (40 μM) in buffer containing 96 μM tubulin (solid curve) and in buffer (broken curve). Compound **2** in pure buffer exhibits no circular dichroism. (c, Right) Absorption and circular dichroism spectra of **3** (35 μM) in buffer containing 90 μM tubulin (solid curve) and in pure buffer (broken curve).

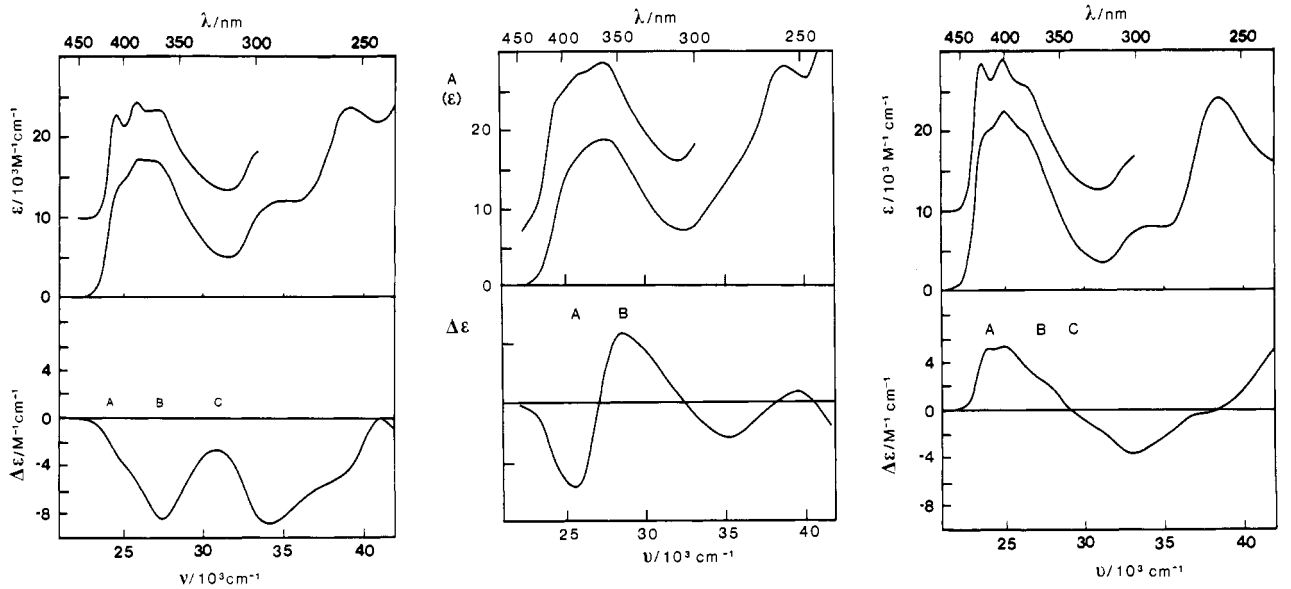

FIGURE 2: Absorption and circular dichroism spectra of thiocolchicine and analogues in ethanol solution at 25 °C. Absorbance given in molar units unless otherwise specified. Absorption spectra of the solutions recorded at −90 °C are also shown (vertically displaced; absorbance in arbitrary units). Apart from a more resolved vibrational structure the CD spectra of **1** and **3** in ethanol at −90 °C do not significantly differ from those at 25 °C. (a, Right) Absorption and CD of **1**. (b, Middle) Upper panel: absorption spectrum of **2**. Lower panel: MCD spectrum of the optically inactive compound **2** in ethanol solution (see text). (c, Left) Absorption and CD of **3**.

37 °C it required approximately 15 min for complete association.

The absorption changes upon binding to tubulin are similar for the three compounds. The intensity of the low-energy band decreases compared to that in buffer solution, and a vibrational structure emerges on the long-wavelength side of this band. For **1** and **2** the band is also slightly red-shifted. The effects on the CD spectra are more contrasting. The tubulin complexes with the three compounds all show a negative CD band at 413–424 nm, with vibrational structure, which we shall denote the A component. Compared to the spectra in buffer, the sign of the A component is the same for **1** but the opposite for **3** in complex with tubulin, while for the optically inactive compound **2** the A component is the only clear CD band that is induced upon binding to tubulin. The next CD band at higher energy, denoted the B component, observed at 368–377 nm, is a positive band in **3** that appears unaltered upon binding. For compound **1**, the B component is a negative band, which upon binding to tubulin is reduced to approximately half its magnitude. With compound **2** a weak and rather broad positive band centered at 342 nm in the complex with tubulin can be tentatively assigned to the B component. Finally, the C component shows up as a positive CD band at 327 nm in the tubulin complex with **1** but is less clear in the buffer spectra owing to spectral overlap with transitions at higher energy. Apparently related is a negative shoulder located at about 330 nm in the buffer spectrum of **3**. It retains its negative sign on binding, but as a minimum on a positive background, due

Table I: Maxima of the Low-Energy Absorption Band[c] of Thiocolchicine and Analogues

compd	solvent[a] cyclohexane	ethanol	ethanol, −90 °C	water	tubulin[b] in buffer
1	403 sh (11.6)	404 sh (14.5)	407 (*d*)		414 (14.7)
	386 (15.8)	385[e] (18.0)	388	383 (18.3)	395 (16.4)
	369 (15.7)	371 (17.9)	369		374 (16.6)
2			405 sh		413 (14.0)
	379 (16.2)		386 sh		392 (16.9)
	355 (16.4)	363 (18.8)	363	366 (19.8)	367 (18.9)
3	418 sh (16.8)	418 sh (22.5)	423	415 sh (24.3)	423 (21.2)
	399 (22.0)	401 (24.8)	402	400 (24.8)	401 (22.8)
	384 sh (19.6)	380 sh (21.5)	382 sh		380 sh (18.8)
	370 sh (17.4)				

[a] Recorded at 25 °C unless otherwise specified. [b] Concentrations and conditions as described in the legend to Figure 1. [c] Wavelength (nm); in parenthesis, ϵ (10^3 M^{-1} cm^{-1}); sh = shoulder. [d] Extinction coefficients were not determined for the spectra obtained at −90 °C. [e] Lit. 385 (17.8) (Shiau et al., 1975), 387 (18.6) (Fabian et al., 1955).

to a sign change of an overlapping band at higher energy.

At low temperature (−90 °C), the more rigid tricyclic compounds **1** and **3** show a vibrational structure in the low-energy band that closely resembles the spectra of the complexes with tubulin at room temperature (Figure 2). This observation indicates a marked conformational immobilization of the chromophore in the bound state, as has also been suggested by Bhattacharyya and Wolff (1984) on the basis of the fluorescence properties of colchicine. There is an energy difference between the two first absorption peaks of about 1200–1300 cm^{-1} for all three compounds when in cold ethanol (−90 °C) or bound to tubulin. This energy difference correlates well with the Raman band at 1288 cm^{-1} reported for the tubulin complex with compound **1** (Rava et al., 1987). None of the three compounds **1**, **2**, and **3** showed any significant fluorescence increase upon binding to tubulin, in contrast to the colchicine series.

Absorption Spectra in Different Solvents. Table I summarizes the extinction coefficients and wavelength positions for the peaks of the first absorption band of compounds **1**–**3** in different solvents and in their complexes with tubulin.

The features of the low-energy absorption band, with respect to wavelength positions of peaks and shoulders, are essentially the same in all of the solvents for the two more rigid compounds **1** and **3**. However, the intensity is significantly decreased on going from water or ethanol to cyclohexane as solvent, and the decrease is most pronounced on the long-wavelength side of the band. Although the extinction coefficient is a rather crude measure of the oscillator strength of the fairly complex low-energy band, a striking observation is the essentially constant difference in the maximum extinction coefficient ($\delta\epsilon$ = 6500 ± 300 M^{-1} cm^{-1}) between **3** and **1** in water, in ethanol, and in cyclohexane as well as in their tubulin complexes. In all solvents the relative intensity increase from **1** to **3** is largest on the long-wavelength side of the band. The more conjugated chromophore system in compound **3** leads to a marked red shift of the low-energy band compared to that for **1** (about 900 cm^{-1} in ethanol at −90 °C).

A comparison of the low-energy band of **1** with that of **2** indicates some pertinent features. In ethanol at −90 °C, the first two absorption maxima in **2** (shoulders at 405 and 386 nm) are found at nearly the same positions as in **1** (407 and 388 nm), whereas the third maximum is found at markedly shorter wavelength in **2** (363 nm) than in **1** (369 nm). In ethanol at 25 °C, the third absorption maximum at 371 nm in **1** is slightly less intense (ϵ = 17 900 M^{-1} cm^{-1}) than the maximum at 363 nm in **2** (ϵ = 18 800 M^{-1} cm^{-1}). The oxo analogues of **1** and **2** have been found to exhibit similar features. In neutral aqueous buffer, colchicine has its absorption maximum at 353 nm (ϵ = 15 950 M^{-1} cm^{-1}; Andreu & Timasheff, 1982b) and MTC at 343 nm (ϵ = 17 600 M^{-1} cm^{-1}; Andreu et al., 1984).

Circular Dichroism Spectra in Different Solvents. Table II collects positions and intensities of the low-energy CD bands determined for **1** and **3** in ethanol and cyclohexane (compound **2** is not optically active) and for the three compounds when bound to tubulin. The CD spectra of **1** and **3** in ethanol solution are shown in Figure 2, together with the magnetic circular dichroism (MCD) spectrum of **2** recorded in ethanol at ambient temperature. A bisignate MCD pattern in the region of the low-energy absorption of **2** is indicative of the presence of two close-lying electric dipole allowed transitions (Nordén et al., 1978).

While the CD spectra of **1** and **3** are quite similar at higher energies, the low-energy CD bands are of opposite sign in ethanol solution. Although the absolute configuration of **3** has not yet been settled, this shows that the axial chirality of the phenyltropone chromophore cannot be related in a simple way to the signs of the CD components. However, the high-energy components, with rotary strength from exciton coupling (Hrbek et al., 1982), can be considered a more reliable indicator of the axial chirality than the low-energy band. Therefore the invariance of the CD features in this spectral region suggests that the chirality of **3** is *a*S, the same as in natural colchicine. Further support for this conclusion is the rapid association of **3** to tubulin at low temperature, knowing that tubulin only accepts colchicinoids with *a*S conformation (Yeh et al., 1988). No evidence for rapid atropisomerization nor significant amounts of the other atropisomer of **3** could be detected by NMR.

The CD spectrum of a saturated cyclohexane solution of **1** (50 μm) shows a positive A component, which changes sign upon the addition of a minute amount (0.2%) of ethanol, but without any accompanying major changes in the rest of the spectrum (Figure 3). The latter observation indicates that the *a*S conformation of **1** is retained. The relative amplitude

Table II: CD Components of the Low-Energy Band[a] of Thiocolchicine and Analogues

compd	conditions	component A		B	C
1	ethanol	406 sh (−3.0)		364 (−8.2)	
	cyclohexane	402 (+3.6)	390 sh (+3.2)	357 (−6.8)	
	tubulin complex	415 (−3.5)	393 (−3.7)	368 (−4.4)	327 (+1.8)
2	tubulin complex	413 (−5.8)	394 (−5.0)	342 (+0.9)	
3	ethanol	416 sh (+5.7)	400 (+6.1)	366 sh (+3.2)	330 sh (−1.0)
	cyclohexane	417 sh (+4.6)	400 (+6.2)	360 sh (+2.3)	320 sh (−2.0)
	tubulin complex	424 (−3.9)	403 (−2.8)	377 (+2.0)	~340 (pos. min.)

[a] Wavelength (nm); sh = shoulder; in parenthesis, $\Delta\epsilon$ (M^{-1} cm^{-1}).

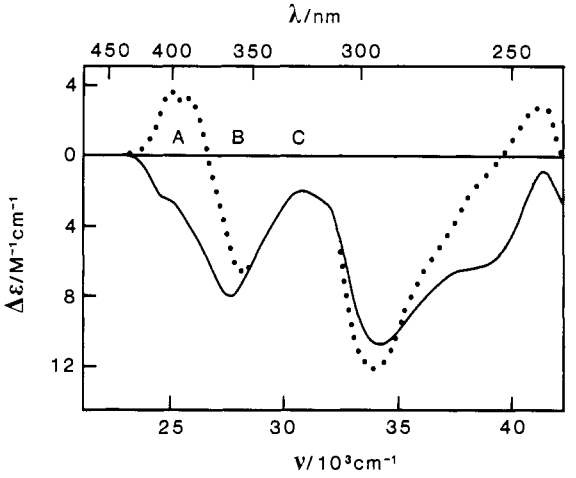

FIGURE 3: Circular dichroism of **1** (50 μM) in pure cyclohexane solution (dotted curve) and after addition of 0.2% ethanol (solid curve).

of the A component is also markedly reduced, although still positive, in more dilute solutions (2 μm). Together with the low solubility, this observation suggests that the positive sign may be an effect of aggregation of **1** in the nonpolar solvent. By contrast, the CD spectrum of **3** in cyclohexane is not much different from that in ethanol.

Interestingly, similar behavior as with **1** is found for colchicine with two low-energy CD bands in pure cyclohexane, at 379 nm ($\Delta\epsilon$ = +1 M^{-1} cm^{-1}) and at 337 ($\Delta\epsilon$ = −5 M^{-1} cm^{-1}), which change into a single negative band at 351 nm ($\Delta\epsilon$ = −8 M^{-1} cm^{-1}) upon the addition of 0.2% ethanol, without any other major changes in the CD spectrum.

Linear Dichroism in PVA Film. The reduced linear dichroism (LD^r) of **1** in stretched PVA film is shown in Figure 4. A marked variation of the LD over the long-wavelength absorption band with a maximum at 350 nm supports the conclusion from MCD that there are two overlapping transitions in this region. A drop in LD^r at about 310 nm further suggests the presence of an additional transition located near the short-wavelength edge of the first absorption band. This transition appears quite weak, however, and is largely hidden in the isotropic absorption envelope.

Figure 4 also shows the "reduced circular dichroism" (CD^r = CD/A) of **1** in ethanol solution at 25 °C. Three approximately constant regions, around 330 and 355 nm and one above 400 nm, correlate nicely with the three CD components, A, B, and C, suggested for the tubulin complex spectra (vide supra) and the three electronic transitions suggested from the LD^r pattern.

Discussion

The Thiocolchicine Chromophore. We find firm indications from MCD, LD, and CD (Figures 2b and 4) that the low-energy band of the compounds studied is due to two electronic transitions, the first of which (A) is characterized by a more or less resolved vibrational structure. A similar conclusion has also been reached by Hastie and Rava (1989) for colchicine from multiple differentiation of the absorption profile. Interestingly, the wavelength maxima of the fourth-derivative absorption band of colchicine in ethanol reported by these authors (379, 362, and 346 nm) display a practically constant energy difference of 1800 cm^{-1} between the peaks of the absorption spectrum of **1** in ethanol at −90 °C (Table I). This correlates well with our CD spectra of colchicine or **1** in cyclohexane solution. The low-energy bands of the two compounds thus appear to be closely related, although comparison

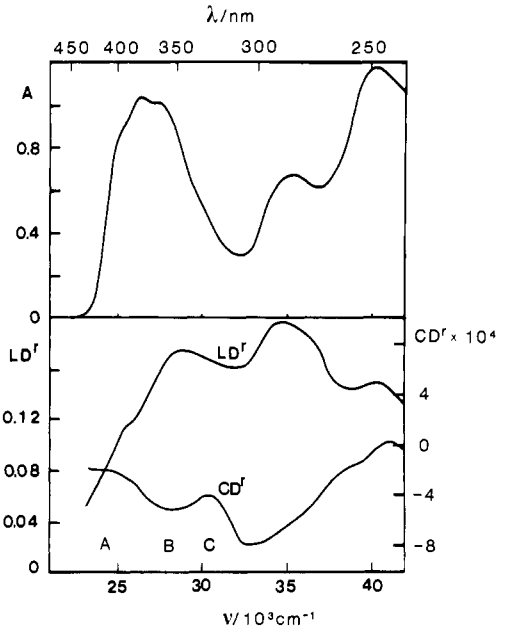

FIGURE 4: Upper panel: isotropic absorption spectrum of **1** in stretched PVA film. Lower panel: LD^r of **1** in stretched PVA film and CD^r of **1** in ethanol solution (see text).

of their absorption spectra indicates the A transition to be of significantly lower intensity in colchicine.

Linear dichroism measurements on tropolone and related compounds in stretched films, as well as SCF MO calculations, suggest two $\pi \rightarrow \pi^*$ transitions, in the low-energy absorption region, polarized approximately perpendicular to each other (Kuroda & Kunii, 1967; Hoshi & Tanizaki, 1970). If the same orientations of the transition dipoles are assumed for colchicine as in the parent chromophore tropolone, one may infer the second transition to be polarized approximately along the long axis of the phenyltropone system from the SCF MO calculations. Furthermore, the first transition is characterized by a transfer of electron density from the tropone carbonyl to the ring and is predicted to be intensified and slightly blue-shifted in polar or protic solvents, which is also observed in the case of colchicine (Hoshi & Tanizaki, 1970; Hastie & Rava, 1989).

The energy of the weak third transition (C), the location of which is indicated from the LD^r and the CD^r profiles (Figure 4), appears too low to be described as a higher tropone or benzene transition and may be tentatively assigned as a transition involving the entire phenyltropone chromophore.

Resonance Raman studies (Rava et al., 1987) and MNDO calculations (Hastie & Rava, 1989) indicate that the low-energy band of colchicine originates primarily from electronic transitions of the tropone moiety. Nevertheless, comparison of the low-energy band (ethanol solution) of 2-methylthiotropone [λ_{max} 343 nm, ϵ = 8600 M^{-1} cm^{-1} (Cavazza et al., 1977)] with that of **1** (λ_{max} 385 nm, ϵ = 18000 M^{-1} cm^{-1}) demonstrates a strong perturbation by the trimethoxyphenyl substituent, which is reinforced in the less twisted compound **3** (λ_{max} 401 nm, ϵ = 24800 M^{-1} cm^{-1}). The B-ring substituent appears to have only little influence on the absorption spectrum, as shown by the virtually identical UV spectra of desacetamidocolchicine and colchicine (Schreiber et al., 1961). This suggests that differences in absorption spectra between **1** and **3** may be ascribed to the different geometries of these chromophores alone. In addition the X-ray crystallographic study of **3** has shown that apart from a dihedral angle ϑ of 30° there is no significant geometrical difference in the chromophoric system as compared to the seven-membered

B-ring thiocolchicinoids (Olsson et al., 1989), where ϑ is about 55° for **1** (Koerntgen & Margulis, 1977). Since simulations of ^{1}H NMR spectra are consistent with the same conformation in solution as in the crystal for seven-membered B-ring colchicinoids (Brossi et al., 1988, Donaldsson, 1988), we shall adopt the crystallographic values of ϑ for **1** and **3** in solution as well.

Absorption Spectra. The absorption spectrum of **3** bound to tubulin is practically superimposable on the spectrum obtained in ethanol at −90 °C, although the extinction coefficient of the complex is closer to that of **3** in cyclohexane than in ethanol. Recalling the apparent insensitivity of the transition energies to solvent polarity, and the absorbance decrease found in nonpolar solvents, we may conclude that **3** binds to tubulin in the same overall conformation as in solution and that the binding site is rather hydrophobic.

From the observation that the difference in maximum extinction coefficients for the tubulin complexes of **3** and **1** coincides with the difference between the free compounds in solution and that the shape of the spectrum of compound **1** in the complex is very similar to its spectrum in ethanol at −90 °C, we may conclude that compound **1** also does not undergo any major conformational change upon binding to tubulin. The red shift found for **1** upon binding to tubulin (about 400 cm^{-1} compared to ethanol at −90 °C) may seem in conflict with this conclusion, unless the binding site perturbs the chromophores of **1** and **3** differently. Since the extinction coefficients of the tubulin complexes of **1** and **3** are already close to the cyclohexane solution values, it seems improbable that a difference in solvation or hydrogen bonding should accidently cancel the expected intensity increase, caused by a significant decrease in ϑ for **1**. We therefore conclude that the colchicine binding site of tubulin is flexible enough to accommodate both **1** with ϑ = 55° and **3** with ϑ = 30°.

Resonance Raman results suggest a ring-stacking interaction between **1** and an aromatic amino acid residue at the binding site (Rava et al., 1987), which is supported by the perturbation observed in the absorbance and fluorescence spectra of allocolchicine by the presence of tubulin (Hastie, 1989).

A nondegenerate coupled oscillator interaction between **1** and an aromatic chromophore of the protein is expected to induce a red shift and decrease the absorption intensity of the lower energy transitions (Cantor & Schimmel, 1980), providing a more consistent explanation for the red shift than a decrease in ϑ. Different orientations of the tropone chromophores of **1** and **3** relative to the interacting chromophore of the protein might account for the different perturbations of their spectra.

For compound **2**, the determination of ϑ in the tubulin-bound state is more difficult, due to the discrepancies between the low-energy bands of **1** and **2** (Table I) and the less well-defined solution conformation of the latter. Its oxo analogue, MTC, crystallizes as an isocolchicine-like rotamer with ϑ = 57° (Rossi et al., 1984). However, since the differences in position of the low-energy bands of **1** and **2** are very similar when bound to tubulin compared to the case in ethanol solution at −90 °C, they are most likely due to the absence of an alkyl substituent on the tropone ring in **2** and not to the presence of the compound as an isocolchicine-like rotamer in the solution. Published absorption spectra of the parent chromophore, 2-(methylthio)tropone, and its 6-isopropyl derivative, show a decreased intensity and a slight blue shift of the low-energy band upon alkyl substitution (Nozoe & Matsui, 1961), in qualitative agreement with the difference between **1** and **2**.

In conclusion, if we can assume that the 5-phenyl-substituted chromophore is affected similarly by a 6-alkyl substituent, we may estimate the dihedral angle of **2** (in ethanol) to be ≥55°, which is in accordance with the conformation of MTC in the crystal and also with molecular mechanics calculations on the latter compound (ϑ = 55°; Berg, unpublished results).

The absorbance spectra of **1** and **2** are both red-shifted by some 400 cm^{-1} upon binding to tubulin, compared to in ethanol at −90 °C. However, in contrast to **1**, the intensity of the low-energy band of **2** does not decrease upon binding (compared to in ethanol). This may reflect an unsymmetrical conformational distribution for **2** in solution, with a bias toward rotamers with larger dihedral angles and less absorbance than the average. This bias is expected since the conformation of **2** is determined by the opposing forces from π-electron interaction (tending to minimize ϑ) and steric repulsion between the 4- or 6-hydrogens and the 2′-methoxy group (tending to increase ϑ). An asymmetric potential (i.e., with decreasing ϑ the repulsion increases more rapidly than the π-stabilization energy) can be anticipated to lead to an unsymmetrical distribution around the average dihedral angle.

A significant decrease in ϑ upon binding of **2** would be expected to alter the shape of the low-energy band, as the intensity increase of this band is most pronounced at longer wavelengths on going from **1** to **3**. However, the band shape is the same for **2**, whether in ethanol at −90 °C or in complex with tubulin, except for a better resolved vibrational structure in the latter case. Since the red shift of **2** upon binding can be explained by the same mechanism as for **1** if the two compounds are bound with a similar geometry, the results are consistent with the same angle ϑ of ≥55° in **2** for the tubulin-bound state and in ethanol solution at −90 °C.

The colchicine binding site has been shown to be essentially bifunctional, with one distinct site for the trimethoxybenzene ring and one for the methoxytropone ring (Andreu & Timasheff, 1982a,b). The bicyclic compound **2**, with a comparatively shallow potential energy surface for rotation around the biaryl bond, can be expected to bind to tubulin with an optimal angle ϑ to fit both sites.

Obviously, compound **3**, with ϑ = 30°, can have less efficient interactions with both of its rings to the two sites. Our results indicate that the interactions with the binding site for the tropone ring differ significantly between **1** and **3**, since the transitions responsible for the low-energy band are largely localized to the 2-(methylthio)tropone chromophore.

In the colchicine series, red shifts of the low-energy absorption upon tubulin binding similar to those we find for **1** and **2** are reported for colchicine (Andreu & Timasheff, 1982b; Hastie & Rava, 1989) and MTC (Andreu et al., 1984), but the intensity does not decrease in contrast to the thiocolchicinoids. The absence of a hydrogen bond to the tropone carbonyl and interaction with an aromatic amino acid residue at the binding site has been proposed to explain the spectral features of colchicine bound to tubulin (Hastie & Rava, 1989).

Circular Dichroism. The low-energy circular dichroism of colchicine has been shown to originate from the chirality of the phenyltropone system rather than from the C-7 chiral center of the B ring (Yeh et al., 1988). Several underlying mechanisms may be considered: (1) chiral perturbation of the tropone chromophore by the trimethoxyphenyl substituent (coupled oscillator CD); (2) intrinsic chirality of the whole phenyltropone chromophore (nonlocalized transitions); (3) intrinsic chirality of the tropone chromophore itself.

The contribution from the first mechanism (1) may be readily calculated from the individual transition properties of

the coupled chromophores. However, as Hrbek et al. (1982) conclude, this mechanism probably plays only a minor role in the low-energy CD band of colchicine. The intrinsic chirality (2) of the phenyltropone chromophore is obvious, but the importance of the second mechanism is difficult to judge, since the low-energy electronic transitions appear to be largely localized to the tropone moiety. It can be expected to be more important for the better conjugated chromophore of **3** than for **1**.

X-ray crystal structures of colchicinoids consistently show a small but significant deviation from planarity of the tropone ring, which usually has a more or less distorted boat conformation (Lessinger & Margulis, 1978; Koerntgen & Margulis, 1977). The asymmetrically substituted tropone ring thus constitutes a potential intrinsic chiral chromophore (3). Although the deviation from planar symmetry is small and energy differences between different tropone ring conformations are quite small in colchicinoids (Donaldsson, 1988), intrinsic chiral chromophores are known to exhibit strong circular dichroism and a mechanism of the third kind has to be considered as well.

Against this background it is interesting to note that the crystal structure of **3** shows a conformation of the tropone ring that differs from those of other colchicine derivatives in the crystal by being what could be described as a distorted chair (Olsson et al., 1989). The difference is most probably an effect of the steric strain between the 11-H and the 1-methoxy group in **3**, whose separation in the molecule is significantly smaller than the sum of their van der Waals radii.

There is no simple correlation between the signs of the low-energy CD components of the thiocolchicine analogues and the sign of their axial chirality (see Table II), which supports the conclusion of Hrbek et al. (1982) that this CD is not due to the first mechanism.

The sign of the A component is clearly independent of both ϑ and the helicity of the system, suggesting that a local dissymmetry of the (methylthio)tropone chromophore is responsible for this CD.

Conclusions

Thiocolchicine, two B-ring-modified analogues, and the corresponding tubulin complexes have been studied by using normal absorption and circular dichroism spectroscopy to examine how conformation and spectroscopic responses are correlated.

From this analysis we conclude that compounds **1** and **2**, when bound to tubulin, have a dihedral angle between the phenyl and tropone planes of about 55°, as found in solution, while the rapid binding of the rigid compound **3** demonstrates that the colchicine site of tubulin may as well accept the more planar conformation of **3** with a dihedral angle of 30°.

The structure and intensity of the absorption spectra suggest that the tropone ring in the three thiocolchicine analogues is immobilized in a nonpolar environment. Compound **3** deviates from the other two compounds with respect to interaction with the colchicine site, as judged from absence of a red shift of the low-energy band.

Neither of the two lowest CD components (A or B), present in all three compounds, appears to be a reliable indicator of the axial chirality of the phenyltropone chromophore. These CD components most likely derive from the local dissymmetry of the tropone ring and seem to be prone to both structural and electronic perturbations.

Acknowledgments

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Registry No. **1**, 2730-71-4; **2**, 130698-87-2; **3**, 124411-92-3; MTC, 60423-21-4; *N*-deacetylthiocolchicine, 2731-16-0; methanethiol, 74-93-1.

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