# Tubulin Binding of Two 1-Deaza-7,8-dihydropteridines with Different Biological Properties: Enantiomers NSC 613862 (*S*)-(−) and NSC 613863 (*R*)-(+)[†]

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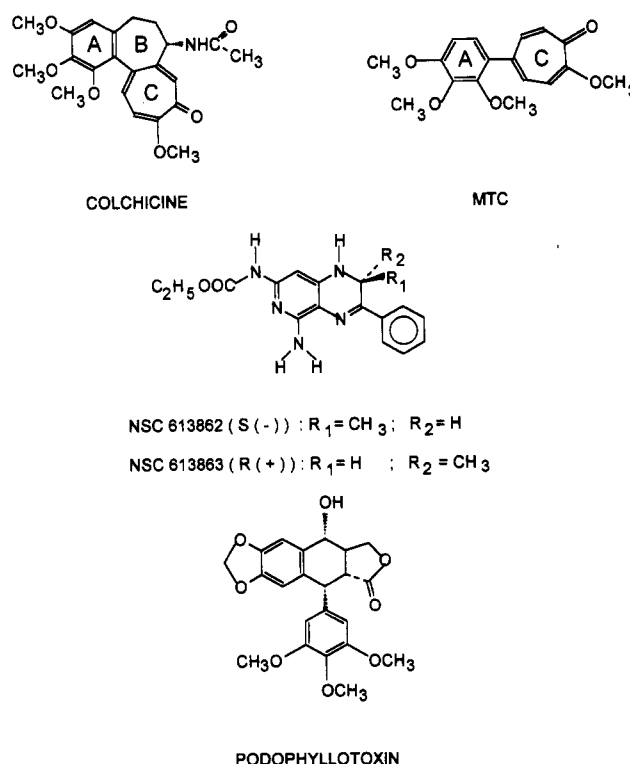
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ABSTRACT: Several fluorescence properties of two enantiomers, NSC 613862 (*S*)-(−) and NSC 613863 (*R*)-(+), have been compared. Even though the two isomers showed the same fluorescence behavior in solution in different solvents, drastic differences were observed after binding to purified calf brain tubulin. Binding measurements for the two compounds were performed both by fluorescence spectroscopy and by column gel permeation, a direct method of measurement. For both isomers, the binding was characterized by the presence of one high-affinity binding site with an apparent association constant of $(3.2 \pm 0.5) \times 10^6$ M$^{-1}$ and $(4.1 \pm 0.9) \times 10^6$ M$^{-1}$ for the *R*- and *S*-isomer, respectively, and by several low-affinity sites. Both isomers were also shown to induce GTPase activity in tubulin. The high-affinity binding site seems to be the same for the two isomers. Moreover, fluorescence competition experiments suggest at least a partial overlap of the colchicine and podophyllotoxin site. To explain the differences in fluorescence behavior after binding to tubulin, we hypothesize that the *R*-isomer is positioned differently in its binding locus as compared with the *S*-isomer.

Initially synthesized by Elliott *et al.* (1968) to act as analogues of folic acid, a number of compounds that possess an aromatic nucleus with a 1-deaza-7,8-dihydropteridine structure have been reported to inhibit mitosis and produce an accumulation of cells in metaphase (Wheeler *et al.*, 1981). Among them, NSC 181928, ethyl (5-amino-1,2-dihydro-3-[(*N*-methylanilino)methyl]pyrido[3,4-*b*]pyrazin-7-yl)carbamate has been studied in particular. Hamel and Lin (1982) demonstrated that this drug was an active antitubulin agent which inhibited both polymerization and binding of colchicine to tubulin and stimulated tubulin-dependent GTP hydrolysis. Bowdon *et al.* (1987) showed that another member of the same series, NSC 370147, ethyl (5-amino-1,2-dihydro-2-methyl-3-phenylpyrido[3,4-*b*]pyrazin-7-yl)carbamate, was able to competitively inhibit the binding of [³H]colchicine and slightly enhance the binding of [³H]vincristine to purified tubulin.

However, NSC 370147 is a racemic compound. Its two chiral isomers, NSC 613862 (*S*)-(−) and NSC 613863 (*R*)-(+) (see Chart I), have displayed significant differences in

Chart I



COLCHICINE

MTC

NSC 613862 ( S (−) ) : R$_1$ = CH$_3$; R$_2$ = H

NSC 613863 ( R (+) ) : R$_1$ = H ; R$_2$ = CH$_3$

PODOPHYLLOTOXIN

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[1] Abbreviations: BSA, bovine serum albumin; PG buffer, 10 mM sodium phosphate and 0.1 mM GTP, pH 7.0; MDL 27048, *trans*-1-(2,5-dimethoxyphenyl)-3-[4-(dimethylamino)phenyl]-2-methyl-2-propen-1-one; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; SDS, sodium dodecyl sulfate; TME, tropolone methyl ether; $\Delta S^c{}_{app}$, difference between the apparent sedimentation coefficient of tubulin alone and liganded.

potency in several biological tests, for example, the determination of their activity against cultured lymphoid leukemia L1210 cells (Temple & Rener, 1989).

The importance of chirality in the behavior of the molecule has been described for colchicine (Yeh *et al.*, 1988; Brossi *et al.*, 1990) (Chart I). In this case, the conformation of the phenyltropolone system in an *aS* configuration is required for binding to tubulin. The *aR* configuration abolishes activity.

The purpose of the present investigation was to describe the analogies and diversities in the interactions of the two enantiomers with tubulin. Spectrofluorometric methods and the gel equilibrium permeation technique were used to study the binding parameters for each enantiomer on purified tubulin. Another part of the study was aimed at determining the binding loci of *R*- and *S*-isomers on tubulin by the use of intensively studied reference compounds like colchicine, MTC [a colchicine analogue], and podophyllotoxin (Chart I).

## MATERIALS & METHODS

*Purified Calf Brain Tubulin.* Calf brain tubulin was purified by ammonium sulfate fractionation and ion-exchange chromatography, stored in liquid nitrogen, and prepared for use as described (Weisenberg *et al.*, 1968; Lee *et al.*, 1973; Andreu *et al.*, 1984). The tubulin–colchicine complex was prepared as described (Andreu & Timasheff, 1982a) and the tubulin concentration in the complex was measured spectrophotometrically using an extinction coefficient of $\epsilon_{275nm} = 1.14 \text{ L g}^{-1} \text{ cm}^{-1}$ in 1% SDS in neutral aqueous buffer (Andreu & Timasheff, 1982a).

*Chemicals.* NSC 613862 (*S*)-(-) and NSC 613863 (*R*)-(+) were synthesized as described by Temple and Rener (1989). Stock solutions were made in dimethyl sulfoxide and stored at -20 °C. Concentrations were measured spectrophotometrically with extinction coefficient values, $\epsilon_{374nm} = 15\ 100$ and $15\ 400\ \text{M}^{-1}\text{cm}^{-1}$ for *S* and *R*, respectively (Temple & Rener, 1989). MTC [2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one] was a gift from Dr. F. J. Fitzgerald (Fitzgerald, 1976), and its concentration was spectrophotometrically measured with the extinction coefficient $\epsilon_{343} = 17\ 600\ \text{M}^{-1}\ \text{cm}^{-1}$ (Andreu *et al.*, 1984). Podophyllotoxin was from Aldrich Chemical Co. and $\epsilon_{290} = 3700\ \text{M}^{-1}\ \text{cm}^{-1}$ was used for the spectroscopic measurements. GTP disodium salt, colchicine, ethylene glycol, and glycerol were from Fluka. [$\gamma$-$^{32}$P]GTP triethylammonium salt (10 mCi/mmol) was from Amersham International.

*Tubulin Concentrations.* Tubulin concentrations were determined spectrometrically at 275 nm in 6 M guanidine hydrochloride ($\epsilon_{275nm} = 1.09\ \text{L g}^{-1}\ \text{cm}^{-1}$) or in 0.5% sodium dodecyl sulfate in neutral aqueous buffer ($\epsilon_{275nm} = 1.07\ \text{L g}^{-1}\ \text{cm}^{-1}$) (Lee *et al.*, 1973; Andreu *et al.*, 1984).

*Spectroscopic Measurements.* Light absorption spectra were obtained with a Beckman DU 70 spectrophotometer. Fluorescence measurements and uncorrected spectra were obtained with a Perkin-Elmer Luminescence Spectrometer 50, monitored by an IBM PS2 computer, with slit widths of 2.5/5 nm and also with a Kontron SFM 25 spectrofluorometer with slit widths of 5/5 nm. Fluorescence spectra were obtained using 1 (excitation direction) $\times$ 0.2 cm cells (Hellma) thermostated at 25 $\pm$ 0.5 °C.

*Binding Measurements by Fluorometric Titration:* (*A*) *Fluorescence of the Ligand.* When the ligands are excited at 380 nm in the presence of tubulin, an increase in fluorescence is observed at about 460 nm, which is the emission wavelength for the bound ligand and to a much smaller extent for the free ligand.

(*B*) *Data Treatment.* The monitoring of the output from the spectrofluorometer and the optimization of the binding and spectral parameters were performed using personal computer programs on a compatible PC-AT (M. Sarrazin, to be described in detail elsewhere). The fluorescence spectra were corrected for the effect of the protein by a simple point-by-point subtraction of the protein spectrum from the spectra of the tubulin plus ligand solutions. The fluorescence intensity is proportional to the concentration over only a limited range of optical densities of the sample at the excitation and emission wavelength. In this range, the increase in the peak at about 460 nm was proportional to both the free [$L_f$] and the bound ligand [$L_b$] concentrations.

(*1*) *Procedure I.* In general the intensity of this signal is given by

$$F_{460} = (A[L_f] + B[L_b])e^{-C[L_0]} \tag{1}$$

where [$L_0$] is the total ligand concentration. If the binding sites on tubulin are of a single class and if *n* is the number of sites and $K_d$ is the apparent dissociation constant

$$K_d = \frac{[L_f](n[P_0] - [L_b])}{[L_b]} \tag{2}$$

where [$P_0$] = total protein concentration. Then eq 2 was solved with respect to [$L_b$]:

$$[L_b] = {}^1\!/_2\{([L_0] + n[P_0] + K_d) - (([L_0] + n[P_0] + K_d)^2 - 4n[P_0][L_0])^{1/2}\} \tag{3}$$

The algorithm starts with an arbitrary opening set of *n* and $K_d$ values. Using these values one can calculate [$L_b$] and [$L_f$] ([$L_f$] = ($L_0$) – [$L_b$]) and then parameters *A*, *B*, and *C*. The initial parameter set is corrected in the next step by a Newton–Gauss procedure (Hamilton, 1964). This iterative procedure is continued until one obtains a minimum sum of squared deviations between experimental and calculated spectra.

(*2*) *Procedure II.* However, since both the fluorescence intensities and the shapes of the titration curves were different for the two enantiomers (see Figure 2 insets), we checked the validity of the above method (procedure I) by using a classical procedure for the correction of the inner filter effect (downward trend of a saturation curve in high-concentration region of the ligand). In the case of the *R*-isomer the fluorescence measurements could be corrected from the inner filter effect by the method of Mertens and Kägi (1979). Then the fluorescence-corrected data ($F_{corr}$) were fitted to the saturation curve equation by means of nonlinear least-squares regression analysis (Johnson & Faunt, 1992):

$$F_{corr} = \frac{F_{max}[L_f]}{K_d + [L_f]} \tag{4}$$

$F_{max}$ is the plateau fluorescence value. Concentrations ([$L_b$] and [$L_f$]) and binding parameters (*n* and $K_d$) were solved using eq 3.

(*C*) *Quenching of the Protein Fluorescence.* Quenching of the intrinsic protein fluorescence by ligand was also employed to estimate the binding parameters. The maximal fluorescence quenching by an excess of ligand was measured and corrected for the inner filter effect by the procedure of Mertens and Kägi (1979). The fraction of sites occupied, *X*, was considered as equal to the fraction of the maximal quenching effect at a given total ligand concentration. The binding equilibrium constant was determined by using

$$1/(1 - X) = -nK_a[P_0] + K_a([L_0]/X) \tag{5}$$

(Bourdeaux-Pontier *et al.*, 1978) where $K_a$ is the apparent equilibrium binding constant.

*Direct Binding Measurements.* The Hummel–Dreyer column gel permeation technique was used to perform binding measurements. A Bio-Gel P4 column (0.9 × 15 cm) was thermostated at 25 °C (isomers adsorb to Sephadex G-25). A syringe pump was used and the flow rate was set at 0.2 mL·min⁻¹. A solution consisting of 9 μM tubulin and a known total concentration of ligand in 0.7 mL of PG buffer was applied to the column preequilibrated with the same buffer of identical ligand concentration. The ligand and protein concentrations were measured spectrophotometrically from the column effluent. To displace the protein–ligand interaction and to solubilize any aggregated protein, we added a small volume of concentrated sodium dodecyl sulfate (final SDS concentration of 1%) to each collected fraction.

The bound and free ligand concentrations were measured at 374 nm; the extinction coefficients in 1% SDS (in neutral aqueous buffer) were 16500 ± 900 and 15500 ± 300 M⁻¹ cm⁻¹ for R and S, respectively. The tubulin concentration was determined at 275 nm in 1% SDS. However, the enantiomers present a nonnegligible absorbance at 275 nm; hence the optical density at 275 nm was corrected for the contribution of bound and free ligand. The absorbance at 275 nm for the bound ligand was calculated by determination of the ratio between the absorbance at 275 nm and absorbance at 375 nm after dissociation in 1% SDS.

The data were interpreted by the use of a nonlinear regression corresponding to

$$[\text{bound ligand}]/[\text{protein}] = \frac{n_1 K_{a_1}[L_f]}{1 + K_{a_1}[L_f]} + \frac{n_2 K_{a_2}[L_f]}{1 + K_{a_2}[L_f]} \quad (6)$$

Equation parameters were $K_{a_1}$, apparent association constant for high-affinity binding sites; $K_{a_2}$, apparent association constant for low-affinity binding sites; $n_1$, number of high-affinity binding sites; and $n_2$, number of low-affinity binding sites.

*Sedimentation Velocity.* Sedimentation velocity experiments were performed with a Beckman Model E analytical ultracentrifuge. Identical samples (10 mM phosphate buffer, 1 mM GTP, pH 7.0, tubulin 9.3 mg·mL⁻¹), with or without ligand, were run simultaneously in double-sector KEL-F cells in a Beckman An-H rotor at 60 000 rpm at 20 °C.

*Ligand-induced GTPase activity* was assayed by the release of [³²P]phosphate in 10 mM sodium phosphate–1 mM MgCl₂ buffer, pH 7.0, containing 0.1 mM [γ-³²P]GTP, at 37 °C [see Peyrot et al. (1992)].

## RESULTS

*Spectral Properties of NSC 613862 and NSC 613863.* For both enantiomers, the absorption spectrum was characterized by two maxima at about 240 and 374 nm and one absorption minimum at 300 nm (Figure 1A, inset). In neutral aqueous buffer, the pyrido[3,4-*b*]pyrazine group also presents two absorption maxima at 230 and 315 nm (Perkampus et al., 1966). The bathochromic shift observed for the enantiomers was induced by the resonance between substituents (see Chart I) and delocalized electrons from the pyrido[3,4-*b*]pyrazine ring (Jaffé & Orchin, 1964).

As shown in Figure 1A, R- and S-isomers present similarly weak fluorescence spectra in neutral aqueous buffer, with maxima at 380 and 480 nm for excitation and emission, respectively. When bovine serum albumin was mixed in a 1:1 protein to isomer ratio and the excitation wavelength was set at 380 nm, a small increase, less than 2-fold, was observed for both enantiomers with respect to their free fluorescence spectra.
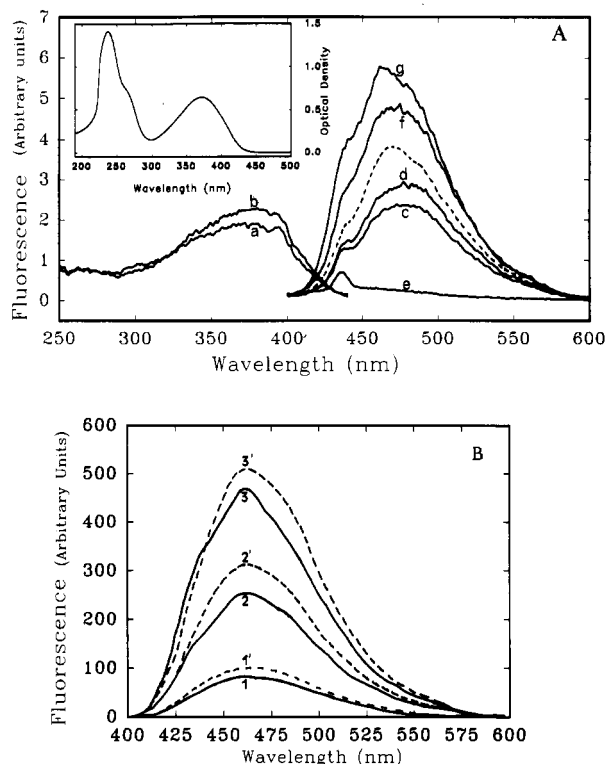


FIGURE 1: (A) Fluorescence changes produced by BSA–isomer interactions. Fluorescence excitation spectra of S-isomer (a) and R-isomer (b) (3 μM) in neutral aqueous buffer (emission at 480 nm) are shown. (c) and (d) are the corresponding emission spectra (excitation 380 nm). Also shown are the emission spectra of a solution containing BSA (3 μM) (e) when mixed with 3 μM of R- (f) or S-isomer (g) at 25 °C. The dashed line represents the emission fluorescence spectrum of S-isomer (3 μM) in solution in ethanol (reduced scale: 1.5-fold). The inset shows the absorption spectrum of the S-enantiomer (43 μM) in PG buffer, pH 7.0, at 25 °C. (B) Fluorescence emission spectra of S (50.4 μM) or R (51 μM) with different solvent viscosities. The excitation wavelength was 380 nm. Ethylene glycol/glycerol mixtures at 25 °C were used: for the S-isomer, (1) 100% ethylene glycol, (2) 3/7 ratio, and (3) 100% glycerol; for the R-isomer, (1') 100% ethylene glycol, (2') 3/7 ratio, and (3') 100% glycerol.

This indicates a probable interaction between bovine serum albumin and the isomers. The fluorescence intensity of the BSA–S complex was slightly higher than that obtained with the R-isomer.

Using several mixtures of ethylene glycol/glycerol of different viscosities, we obtained an increase in the emission fluorescence spectrum intensity for both enantiomers (see Figure 1B). The range of viscosity used was from η = 49 cP (ethylene glycol) to η = 391 cP (glycerol). In this experiment the increment in the dielectric constant of ethylene glycol and glycerol at 25 °C was very weak (from 37.7 to 42.5). For both enantiomers, the fluorescence quantum yield increased with viscosity but did not affect the protein maximum of the emission wavelength.

The effect of the solvent dielectric constants on the isomer fluorescence emission spectra, at 25 °C, was also tested with solvents having a similar viscosity (ca. 0.900 cP). For both enantiomers the intensity of the fluorescence emission spectra in carbon tetrachloride (ε = 2.23) was only 2-fold higher than that obtained in water (ε = 78.3) and remained very weak. A blue shift was also observed for the maximum wavelength when the polarity was decreased.

*Binding to Tubulin.* Examination of the uncorrected fluorescence spectra of the free R-isomer and R mixed with tubulin showed (see Figure 2A) that the free ligand (3 μM)
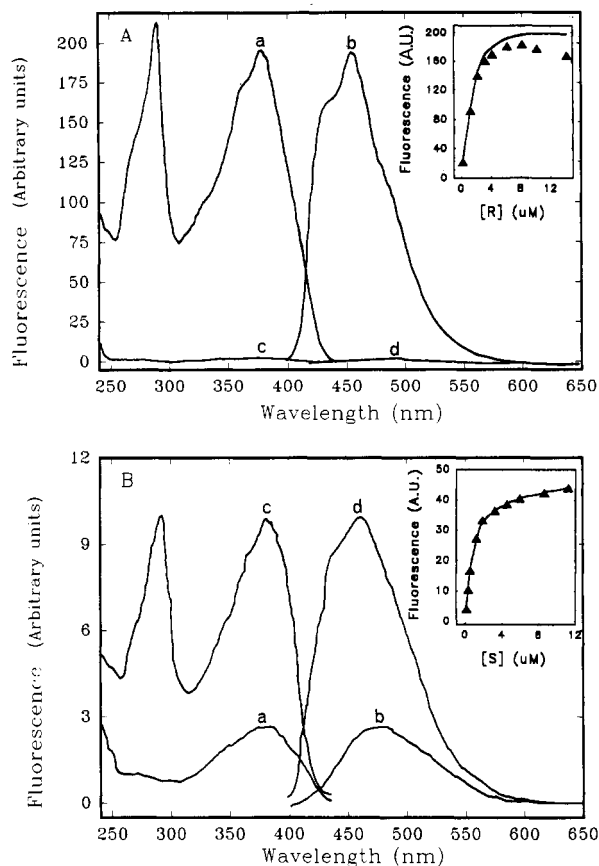
FIGURE 2: (A) Fluorescence changes induced by tubulin-$R$ complex formation at 25 °C. Uncorrected excitation (emission 460 nm) (a) and emission (excitation at 380 nm) (b) fluorescence spectra were obtained after addition of tubulin (3.5 $\mu$M) to the initial solution consisting of 3.5 $\mu$M $R$-isomer in PG buffer (c and d). The inset shows the fluorometric titration of 3 $\mu$M tubulin with the $R$-isomer; the triangles are the experimental points and the solid line is the fit obtained as described in Materials and Methods. (B) Fluorescence changes induced by tubulin-$S$ complex formation at 25 °C. Uncorrected fluorescence excitation (a) (emission 460 nm) and emission spectra (b) (excitation at 380 nm) of a solution consisting of 3 $\mu$M $S$-isomer in PG buffer are shown. Spectra (c) and (d) were obtained after addition of tubulin (3 $\mu$M) to the initial solution. The inset shows the fluorometric titrations of 2 $\mu$M tubulin with the $S$-isomer; the solid line is the fit obtain as described in Materials and Methods.

exhibited a negligible fluorescence as described above, whereas the bound ligand was markedly fluorescent, with excitation maxima at about 290 and 380 nm and an emission maximum at 457 nm. There was a more than 60–70-fold increase in fluorescence intensity of the $R$–tubulin complex with respect to free $R$ (see inset to Figure 2A). The uncorrected emission spectrum of the $R$ compound shifted to shorter wavelengths, from 480 to 457 nm, upon binding to tubulin. The peak at about 290 nm suggests an energy transfer from aromatic residues of the protein to the bound ligand. Similar results were obtained for the $S$-isomer with or without tubulin (see Figure 2B), but in contrast, the fluorescence intensity in the presence of tubulin was increased about 4-fold. The blue shift upon mixing enantiomers with tubulin may result from isomer binding to an apolar region of the protein and/or from an "immobilization" of the chromophoric system.

*Ligand Fluorescence Titrations of Tubulin.* The insets of Figure 2 show the titrations of a constant tubulin concentration with various concentrations of $R$- and $S$-isomers. At this step some observations must be made: for the $R$-isomer, a typical saturating titration curve was observed (see Figure 2A, inset); in contrast, for the $S$-isomer a nonsaturated titration curve
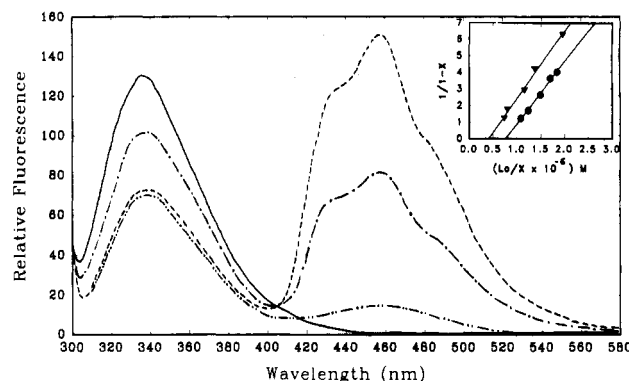


FIGURE 3: Fluorescence changes in the protein emission spectra produced by $S$- and $R$-enantiomers (excitation at 295 nm): (—) tubulin, 3 $\mu$M in PG buffer, pH 7.0; (- ·· -) 8.2 $\mu$M $S$-isomer; (- · ·) 1.2 $\mu$M $R$-isomer; (- - -) 10.2 $\mu$M $R$-isomer. The inset shows the protein fluorescence quenching titration produced by $R$- (circle) and $S$-isomer (triangle) binding to 2.0 $\mu$M tubulin at 25 °C (emission wavelength was 335 nm).

was obtained (see Figure 2B, inset). The data for both isomers were analyzed using nonlinear regressions (see Materials and Methods, procedure I). The apparent binding parameters obtained were $K_a = (2.7 \pm 0.4) \times 10^6$ and $(3.0 \pm 0.6) \times 10^6$ M$^{-1}$ and $0.60 \pm 0.08$ and $0.65 \pm 0.10$, for the apparent binding constant and the number of sites for $R$ and $S$, respectively. The results obtained for the $R$-isomer, employing procedure II, were within experimental error: $K_a = (3.1 \pm 0.6) \times 10^6$ M$^{-1}$ and $n = 0.68 \pm 0.15$.

*Fluorescence Quenching of Tubulin by the R and S Enantiomers.* As shown in Figure 3, when the excitation wavelength was at 295 nm in order to specifically excite the tubulin tryptophanyl residues, $R$– and $S$–tubulin interactions were manifested by a decrease in tubulin emission fluorescence at 335 nm and an increase in fluorescence intensity at 457 nm. The increase at 457 nm, however, was weaker when $S$ was bound to tubulin than that accompanying $R$ binding. The spectra displayed an isoemissive point at 404 nm for the $R$-isomer and at 416 nm for the $S$-isomer. The potential heterogenous binding suggested by the $S$ fluorescence titrations was prevented by using a range of lower concentrations in ligand (0.2–1.3 $\mu$M) with respect to tubulin (2 $\mu$M) for the calculation of the isomer binding parameters. The inset shows the protein fluorescence quenching (2.0 $\mu$M) titration with the $S$- and $R$-isomers (at least four protein preparations were used). The analysis, performed as described in Materials and Methods, gave an apparent association constant of $(4.14 \pm 0.12) \times 10^6$ M$^{-1}$ for the $S$-isomer and $(3.3 \pm 0.08) \times 10^6$ M$^{-1}$ for the $R$-isomer. The number of binding sites for the $S$- and $R$-isomers were, respectively, $n = 0.52 \pm 0.05$ and $0.43 \pm 0.06$. Even though the binding parameters of both isomers were relatively well established for the high-affinity binding site by fluorometric methods, the class of low-affinity binding sites evoked (see Discussion) required the use of a direct procedure.

*Binding Equilibrium Parameters by Gel Chromatography.* The Hummel and Dreyer (1962) gel permeation technique was used to measure binding of the drugs to tubulin. The technique is applicable only to non-self-associating systems. It is, therefore, imperative to establish whether the binding reaction is linked to protein–protein interactions (Timasheff *et al.*, 1991). The association state of tubulin may be deduced directly from the sedimentation velocity Schlieren profile. A number of compounds, like vinblastine, are known to induce or facilitate the self-association of tubulin (Na & Timasheff, 1980a,b). They showed that vinblastine/tubulin interactions
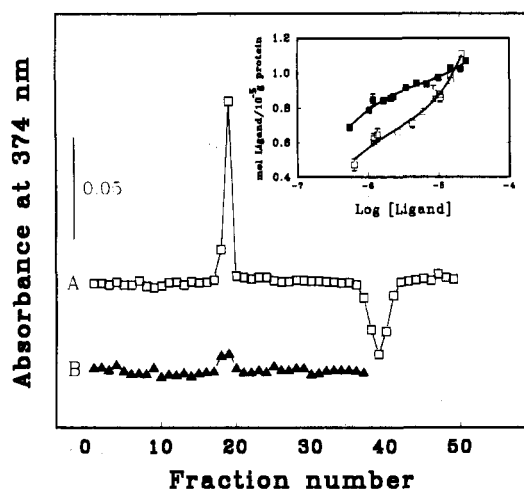
FIGURE 4: Hummel and Dreyer gel permeation of tubulin. (A) When the column was preequilibrated with 4.2 $\mu$M of free R-isomer in PG buffer, pH 7.0, 25°C, a typical elution profile was obtained after chromatography of 0.7 mL of solution containing 9.3 $\mu$M tubulin. (B) A total of 0.7 mL of solution containing 5.5 $\mu$M tubulin was passed through a column equilibrated with 5.6 $\mu$M R and 200 $\mu$M podophyllotoxin. Absorbance measurements were corrected as described in Materials and Methods. The inset shows the binding isotherms of the R- and S-isomers to tubulin in PG buffer, pH 7.0, 25 °C. Open squares are the column measurements obtained with the R-isomer. Filled squares correspond to the column measurements obtained with the S-isomer. The solid lines are the fits to the column measurements.

induced the formation of typical bimodal profiles consisting of different tubulin polymers. Such changes were not obtained upon addition of 240 $\mu$M of R- or S-enantiomer to a 9.3 mg·mL$^{-1}$ tubulin solution in PG buffer at 20 °C. The $\Delta S^c{}_{app}$ values obtained between tubulin and tubulin–isomer were 0.03 S and 0.10 S for R and S, respectively. The gel chromatography technique could therefore be used.

The elution profile (Figure 4, profile A) was characteristic of the technique, i.e., the peak was preceded by baseline and followed by a trough. The region between the peak and trough had a zero concentration increment, which indicated that the binding equilibrium had been reached. For both enantiomers, the Scatchard binding isotherms obtained were curved (see Figure 4 inset). These results were analyzed using the equation for two independent classes of binding sites (see Materials and Methods).

The numerical values of the binding equilibrium parameters obtained after fitting were estimated for the higher affinity at $K_{a_1} = (5.20 \pm 0.86) \times 10^6$ M$^{-1}$ and $n_1 = 0.94 \pm 0.03$ for the S-isomer and at $K_{a_1} = (3.88 \pm 1.61) \times 10^6$ M$^{-1}$ and $n_1 = 0.69 \pm 0.06$ for the R-isomer; for the lower affinity the parameters were estimated at $K_{a_2} = (2.45 \pm 18.4) \times 10^3$ M$^{-1}$ and $n_2 = 8.32 \pm 5.82$ for the R-isomer and at $K_{a_2} = (1.91 \pm 30.1) \times 10^3$ M$^{-1}$ and $n_2 = 2.89 \pm 4.57$ for the S-isomer. These results gave evidence that the binding system of both isomers includes two distinct binding classes. The results for the lower affinity, although meaninglessly imprecise, were required to fit the data properly. Values were consistent with nonspecific binding.

*Specificity of the Interaction.* The results obtained by Bowdon et al. (1987) suggested that 1-deaza-7-8-dihydropteridine inhibits tubulin polymerization by binding to or near to the colchicine site. It was of interest to know whether (i) each enantiomer of NSC 370147 was specifically directed to the colchicine binding site or subsite and (ii) whether both had the same binding site.

The scheme of colchicine binding to tubulin displays two steps. The first is the rapid and reversible formation of a
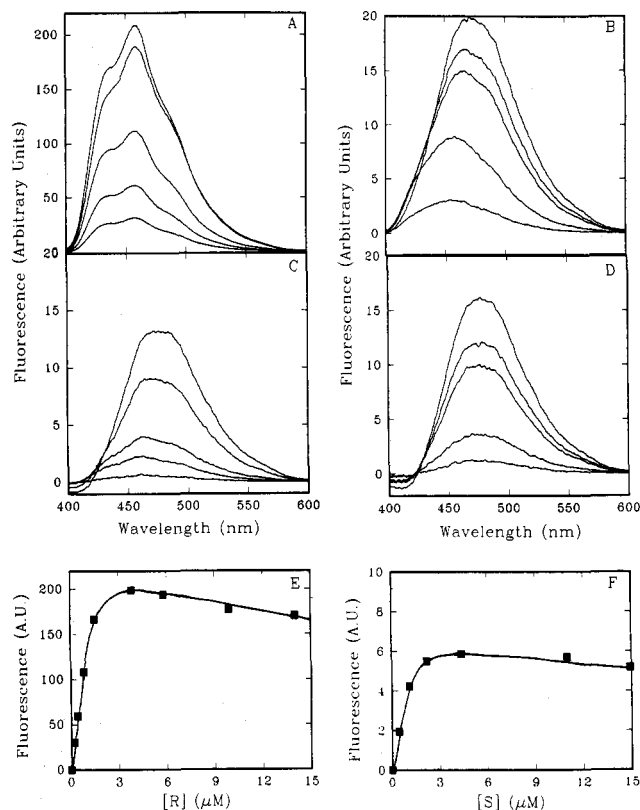


FIGURE 5: Fluorescence emission spectra of 0.2, 0.4, 0.8, 3.8, and 9.9 $\mu$M R-isomer in the presence of 2.6 $\mu$M tubulin (panel A) or 2.7 $\mu$M tubulin–colchicine (panel C), and fluorescence emission spectra of 0.44, 2.2, 11.4, 15.4, and 37.4 $\mu$M S-isomer in the presence of 2.6 $\mu$M tubulin (panel B) or 2.7 $\mu$M tubulin–colchicine complex (panel D). The differences between the titration curves obtained with tubulin and with tubulin liganded to colchicine in the presence of the R- and the S-isomer are shown in panels E and F, respectively. The difference was calculated at 460 nm, which is the maximum emission wavelength for bound enantiomers.

weak tubulin–colchicine complex. The second is a slow step that involves the conformational rearrangement of the protein–ligand complex, leading to a stable final complex (Garland, 1978). According to Diaz and Andreu (1991), the dissociation of tubulin–colchicine complex is a single first-order process with a net rate constant of $3.37 \times 10^{-6}$ s$^{-1}$ at 25 °C. Over the duration of the experiments (1 h) less than 2% of the initial complex was dissociated. Thus the complex can be considered as "irreversible".

Figure 5C,D shows the interaction of the S- and R-isomers with the tubulin–colchicine complex. The emission fluorescence spectra obtained with colchicine-free tubulin are shown in Figure 5A,B. As expected for the R-isomer, we observed a large decrease in the fluorescence emission at 457 nm (compare panels A and C in Figure 5). However, the weak fluorescence obtained was higher than that of the free ligand. Similar findings were obtained with the S-isomer as shown in panels B and D.

The low fluorescence titration values of colchicine-liganded tubulin with both enantiomers were the consequence of the presence of low-affinity binding sites. This observation confirms the lack of clear saturation observed earlier for the S-isomer (see inset to Figure 2B). We therefore used a new approach to determine the apparent equilibrium binding parameters of R and S. The difference between the titration values of tubulin liganded and unliganded with colchicine was attributed to the specific binding of R- and S-isomers to the colchicine site. This difference is shown in panels E and
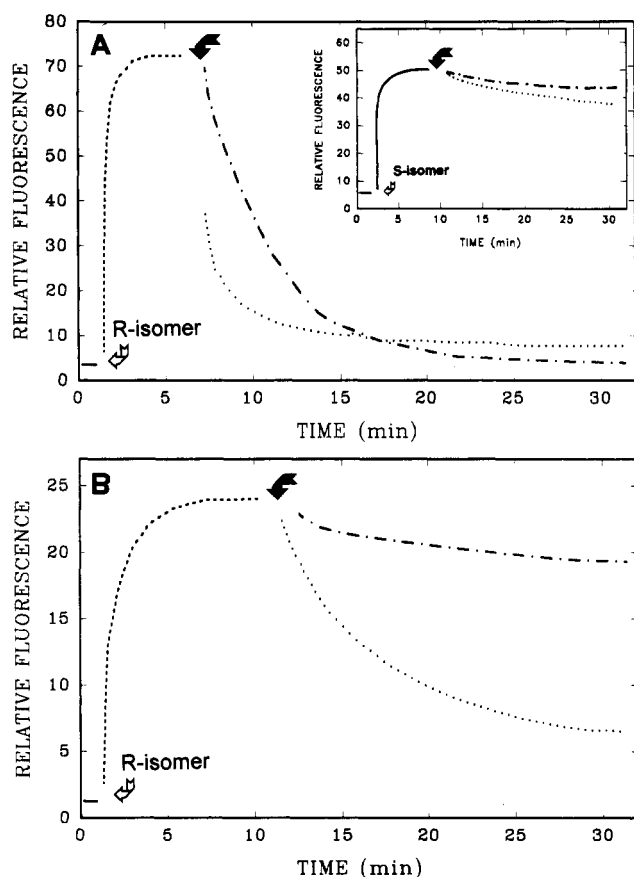
FIGURE 6: (A) Time course of the fluorescence change of 2 $\mu$M *R*-isomer to 3 $\mu$M tubulin at 25 °C (open arrow); at the time indicated by the solid arrow, 200 $\mu$M podophyllotoxin (- - -) or MTC (···) was added. The inset shows the time course of fluorescence change of 1 $\mu$M *S*-isomer added to 2 $\mu$M tubulin. The solid arrow indicates the addition of 200 $\mu$M of podophyllotoxin (···) or 600 $\mu$M MTC (- - -). (B) Changes produced by addition of 0.2 $\mu$M (- - -) or 10 $\mu$M (···) *S*-isomer to 1 $\mu$M *R*-isomer in the presence of 0.7 $\mu$M tubulin.

F of Figure 5. The downward trend observed in the titration curves for high ligand concentrations was due to the contribution of the inner filter effect. After correction of the inner filter effect by the Mertens and Kägi method (see Materials and Methods, procedure II) or by procedure I, data analysis gave the following parameters: for the *R*-isomer, $K_a$ = (3.5 ± 0.2) × 10$^6$ M$^{-1}$ and $n$ = 0.60 ± 0.1; for the *S*-isomer, $K_a$ = (4.0 ± 0.35) × 10$^6$ M$^{-1}$ and $n$ = 0.55 ± 0.09. These results agree with those obtained with the gel chromatography measurement of the protein–ligand interaction on the high-affinity binding site.

Column binding experiments, as described above, were also performed to characterize the specificity of the binding. In this case tubulin was replaced by the tubulin–colchicine complex. In the elution peak, a small optical density increment of ligand (*R*- and *S*-isomers) was observed. This indicates that the binding of the two enantiomers (14 $\mu$M) to the tubulin–colchicine complex (7.6 $\mu$M) was partially inhibited ($\bar{\nu}$ = 0.35 ± 0.02). When the column was equilibrated with podophyllotoxin (200 $\mu$M), the $\bar{\nu}$ value was 0.18 ± 0.06 (Figure 4, profile B).

The locus of isomer binding on tubulin was probed thoroughly by using well-known colchicine competitors. The association kinetics of *R* and *S* to tubulin are presented in Figure 6A. When the equilibrium state of binding was reached (fluorescence plateau), podophyllotoxin or MTC was added. A 100-fold excess of MTC or podophyllotoxin induced a nearly total decrease in the fluorescence signal of the tubulin–*R*

complex in less than 25 min. Inversely, for the *S*–tubulin complex (inset, Figure 6A), 30 min in the presence of a 200-fold excess of podophyllotoxin led to only a 10% decrease in fluorescence intensity. When a 600-fold excess of MTC was used, this decrease was 25% over the same time period. The residual fluorescence was due to the nonspecific binding and to the unbound ligand in both cases.

Finally, it was of interest to know whether the two enantiomers bind to tubulin at the same site. Figure 6B shows that addition of a 10-fold excess of *S* to the *R*–tubulin complex displaced the *R*-enantiomer in less than 25 min.

Tubulin freed of contaminant enzyme activity (see Materials and Methods) can present a GTPase activity induced by some ligand binding (David-Pfeuty et al., 1979; Andreu & Timasheff, 1981). This enzyme activity was induced by colchicine but not by podophyllotoxin. The two ligands were used as references in our experiments in order to determine whether *R* and *S* bound isomers induced a release of inorganic phosphate by tubulin. The GTPase activities, expressed in comparison with 100% GTPase activity of colchicine, were 50% ± 6% and 29% ± 4% for *S*- and *R*-isomer, respectively. No activity was detected for podophyllotoxin.

## DISCUSSION

*Fluorescence Properties of Isomers upon Binding to Tubulin.* Structurally, the *R*- and *S*-isomers differ by the orientation of the methyl group at position 2 relative to the plane of the molecule (see Chart I). They are characterized by very similar absorption and fluorescence spectra when dissolved in neutral aqueous buffer. Their fluorescence emission intensenties are very weak in some solvents and consequently seem weakly dependent on the dielectric constant. Some microtubule inhibitors, such as vinblastine (Wilson et al., 1970) and MDL 27048 (Peyrot et al., 1989), which bind to tubulin, also interact with other proteins. Examination of isomer binding to BSA indicated a weak enhancement of the fluorescence emission spectrum of each isomer. A large increase in the emission fluorescence can be obtained for both enantiomers by raising the viscosity of the solvent. On the other hand, a dramatic increase in fluorescence is provoked by binding of the *R*-isomer to tubulin. This was not observed for the *S*-isomer. Similar phenomena were described by Kung and Reed (1989) for 9-(dicyanovinyl)julolidine (DCVJ) and by Peyrot et al. (1992) for MDL 27048. Restriction in the internal rotation of aromatic groups by high viscosity media or binding to tubulin induced fluorescence enhancement of DCVJ and MDL 27048. In our case, restriction of the free rotation of the phenyl group may explain the viscosity and the binding enhancement of fluorescence observed for the *R*-isomer. However, for the *S*-isomer, this internal rotation would not be equally affected by binding to tubulin. Considering that the free rotation of the phenyl group was apparently more hindered for the *R*-isomer upon binding to tubulin, one may conclude that it is confined differently in its binding locus as compared with the *S*-isomer. Other effects, however, should not be ruled out. For example, Uyeda and Rabinowitz (1963) showed that, for tetrahydrofolate, the fluorescence intensity is a direct function of the ionization state of the N-10 atom. Another example, for colchicinoids, is described by Pyles et al. (1992), who demonstrated the formation of an internal exciplex and, in the presence of glycerol, hydrogen bonding.

Both enantiomers were found to be quenchers of tubulin fluorescence when the protein was excited at 295 nm. As described for colchicine (Andreu & Timasheff, 1982a), the

Table I:   Binding of Isomers to Tubulin at 25 °C

| procedure | isomer | $K_{a_1} \times 10^{-6}$ (M$^{-1}$) | $n_1{}^a$ | $K_{a_2} \times 10^{-6}$ (M$^{-1}$) | $n_2{}^b$ |
|---|---|---|---|---|---|
| ligand fluorescence | S | 3.0 × 0.6 | 0.60 × 0.08 | nd$^c$ | nd |
| | R | 2.7 ± 0.4 | 0.65 ± 0.10 | nd | nd |
| protein fluorescence quenching | S | 4.14 ± 0.12 | 0.52 ± 0.05 | nd | nd |
| | R | 3.30 ± 0.08 | 0.43 ± 0.06 | nd | nd |
| gel permeation | S | 5.20 ± 0.86 | 0.94 ± 0.03 | (1.91 ± 30.1) × 10$^3$ | 2.89 ± 4.57 |
| | R | 3.88 ± 1.61 | 0.69 ± 0.06 | (2.45 ± 18.4) × 10$^3$ | 8.32 ± 5.82 |
| ligand fluorescence competition | S | 4.0 ± 0.35 | 0.55 ± 0.04 | | |
| | R | 3.5 ± 0.20 | 0.60 ± 0.07 | | |

$^a$ Number of high-affinity binding sites. $^b$ Number of low-affinity binding sites. $^c$ Information not determined by this procedure.

compounds may bind near the tryptophanyl residues. A variety of processes can result in quenching, but for the R and S isomers an energy transfer was noted.

*The Tubulin–Isomer Interaction.* As described above and as for colchicine (Andreu & Timasheff 1982), the R and S compounds were able to quench the protein fluorescence with apparent binding constants of about $4.0 \times 10^6$ M$^{-1}$ for S and $3.0 \times 10^6$ M$^{-1}$ for R. A dramatic increase in fluorescence, as reported for colchicine (Arai & Okuyama 1975), appears upon binding to tubulin for the R-isomer, but the fluorescence remained very weak for the S-isomer. The weak fluorescence caused greater difficulties in data treatment for S, as described in Results.

Although the isomer binding parameters agreed with those obtained by quenching measurements, the presence of more than one binding site per tubulin dimer could not be excluded because the S-isomer titration curve never reached the plateau. Indeed, direct binding measurement by equilibrium gel chromatography confirmed, for both enantiomers, the $K_a$ value obtained by the indirect binding measurement for the high-affinity binding site and clearly established the presence of low-affinity sites.

In addition to the one high-affinity binding site, which is able to mediate the aggregation of tubulin (Na & Timasheff, 1980a,b), vinblastine has several other sites with much lower affinity (Wilson, 1975; Wilson et al., 1978; Na & Timasheff, 1986). Low-affinity binding sites have been also described for colchicine (Ringel & Sternlicht, 1984). In both cases, however, these sites seemingly have no effect on the properties of tubulin. The results obtained by analytical ultracentrifugation indicated that both isomers behave like colchicine toward tubulin, i.e., without inducing aggregation. The apparent binding constants to the tubulin dimer high-affinity site of both enantiomorphs were higher (see Table I) than those of the vincaalkaloids ($4 \times 10^4$ M$^{-1}$) (Na & Timasheff, 1986) and of the colchicine analogue MTC ($4.9 \times 10^5$ M$^{-1}$) (Andreu et al., 1984) but very close to that of podophyllotoxin ($2 \times 10^6$ M$^{-1}$) (Cortese et al., 1977) (Garland, 1978), MDL 27048 ($2.75 \times 10^6$ M$^{-1}$) (Peyrot et al., 1992), and colchicine ($5.8 \times 10^6$ M$^{-1}$) (Diaz & Andreu, 1991).

*Characterization of the Isomer Binding Site.* The presence of tubulin–colchicine complex (Figure 5, profile B) or of a large excess in podophyllotoxin inhibited the binding of R- and S-isomers. Colchicine and podophyllotoxin possess a trimethoxybenzene ring which probably participates in the binding to tubulin (Andreu & Timasheff, 1982). The decrease in the tubulin–R complex fluorescence time course induced by the addition of an excess of S-isomer or MTC suggests that both isomers bind to the same site. Both isomers were found to elicit GTPase activity in tubulin. The activity was induced by a tubulin conformational change (David-Pfeuty et al., 1979; Lin & Hamel, 1981). Andreu et al. (1991) showed for colchicine and its analogues that binding to both ring A and C subsites seems to be required to provoke GTPase activity.

The fact that the S-isomer was a better GTPase activity inducer than the R-isomer argues in favor of a higher S-isomer affinity for tubulin. The enantiomers overlap at least one part of the colchicine binding site: the ring A locus, which is apparently common to colchicine and podophyllotoxin. Another part of the tubulin dimer is probably involved in the isomer binding to tubulin, thus explaining the protein quenching fluorescence observed with the isomers but not with podophyllotoxin. Indeed, preliminary results obtained using tropolone methyl ether as a blocking agent of the ring C subsite seem to indicate an inhibition of S- and R-isomer bindings (P. Barbier, personal communication). Then, both subsites of the colchicine binding site may be involved in the S- and R-enantiomer binding.

Both isomers belong to a group of compounds reported to bind with high affinity to tubulin and are competitive inhibitors of the colchicine site without structural likeness to colchicine, e.g., MBC (methylbenzimidazol-2-yl carbamate) (Davidse & Flach, 1977) or more recently Tn-16 (Roach & Luduena, 1985).

In conclusion, both enantiomers bind to one high-affinity binding site on tubulin, with a higher apparent affinity constant for the S-isomer. They also possess low-affinity binding sites. Competition reactions suggested that the R and S isomers bind to the colchicine ring A and C subsites. Furthermore, a tryptophanyl residue is also involved in the isomer binding. The difference in fluorescence observed between the R- and S-isomers can be explained by a different interaction of the isomer fluorophore group with the tubulin site or by the different ionization states of the two isomers when they are bound to tubulin. The differences in the interaction seem (i) to play a major role in the internal rotation of the substituents and/or in the electronic structure of the bound ligand, (ii) to be energetically in favor of the S-isomer binding, and (iii) to be involved in the ligand-induced tubulin GTPase activity.

Colchicine and some colchicine analogues have been reported to promote an abnormal polymerization of tubulin (Andreu et al., 1991). This phenomenon seems to require a particular binding geometry on tubulin. The study of the ability of the isomers to mimic colchicine or colchicine analogues is now in progress. The results will give us a better understanding of the tubulin isomer binding site.

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