Differential Effects of Ethyl 5-Amino-2-methyl-1,2-dihydro-3-phenylpyrido[3,4-*b*]pyrazin-7-yl Carbamate Analogs Modified at Position C_2 on Tubulin Polymerization, Binding, and Conformational Changes

Pascale Barbier,[‡] Vincent Peyrot,*[,‡] Marcel Sarrazin,[‡] Gregory A. Rener,[§] and Claudette Briand[‡]

Proteines et Cancer, URA-CNRS 1924, Faculté Pharmacie, 27 Boulevard Jean Moulin, F-13385 Marseille Cedex 5, France, and Organic Chemistry Research Department, Southern Research Institute, P.O. Box 55305, Birmingham, Alabama

Received July 24, 1995; Revised Manuscript Received October 23, 1995[⊗]

ABSTRACT: NSC 613863 (*R*)-(+) and NSC 613862 (*S*)-(−) (CI980) are two chiral isomers of ethyl 5-amino 2-methyl-1,2-dihydro-3-phenylpyrido[3,4-b]pyrazin-7-yl carbamate which have potent antitubulin activity. The S-isomer is a more potent antimitotic compound than the R-isomer, and the two isomers differ markedly in binding to tubulin [Leynadier, D., Peyrot, V., Sarrazin, M., Briand, C., Andreu, J. M., Rener, G. A., & Temple, C., Jr. (1993) *Biochemistry 32*, 10675−10682]. To understand the origin of such differences, we studied the interactions of three R- and S-isomer structural analogs which differ in C_2 (the chiral carbon), i.e., C179, NSC 337238, and NSC 330770. C179 is a methylated dehydrogenated achiral compound. It bound to tubulin with an apparent affinity K_a of $(2.29 \pm 0.17) \times 10^4$ M^{-1}, inhibited tubulin polymerization *in vitro* at a half-inhibitory concentration (IC_{50}) of 100 μM, and presented no GTPase activity. The substitution of $-CH_3$ by -H leads to the NSC 337238 compound. It bound to tubulin with a higher affinity [$K_a = (2.62 \pm 0.35) \times 10^5$ M^{-1}] and inhibited tubulin polymerization at a lower concentration ($IC_{50} = 14$ μM). It presented no GTPase activity and induced the formation of abnormal polymers at a protein critical concentration (Cr) of 2 mg mL^{-1}. NSC 330770, a demethylated hydrogenated molecule, interacted strongly with tubulin [$K_a = (3.30 \pm 0.56) \times 10^6$ M^{-1}]. It was a potent tubulin polymerization inhibitor ($IC_{50} = 2$ μM) and elicited GTPase activity and the formation of abnormal polymers at a low critical concentration (Cr = 0.5 mg mL^{-1}). Competition experiments showed that the three molecules bound on the colchicine site and the ring A and C subsites. The thermodynamic parameters revealed that, for the most active compounds, i.e., the S-isomer and NSC 330770, many structural changes occurred when they interacted with tubulin. Theoretical chemistry calculations by CNDO/2, correlated with the biochemical activities described above, led us to conclude that the position of the methyl group is not important for activity. Thus, NSC 330770 is a powerful inhibitor which has no methyl group in C_2. Finally, for the molecules to have potent antitubulin activity, they require a 1,2-dihydro structure, a large dipole moment value, and an electron density in the C_3, N_4, and C_{10} atoms.

New derivative series of antimitotic compounds with 1,2-dihydro-3-phenylpyrido[3,4-*b*]pyrazin-7-yl carbamate structure have been tested for biological effects. The compounds showed potent cytotoxicity against cultured L1210 cells and significant anticancer activity against lymphocytic leukemia P-388 in mice, as revealed by the accumulation of cells in mitosis (Temple *et al.*, 1982). NSC 370147, a racemic mixture, was the most active compound (Browdon *et al.*, 1987). Its two chiral isomers, NSC 613863 (*R*)-(+)[1] and NSC 613862 (*S*)-(−) (CI980) (see chart 1), presented differences in biological tests (Temple & Rener, 1989). The S-isomer is the more potent antimitotic inhibitor and is presently in clinical trials (Phase II) (Leopold *et al.*, 1993). The interaction of the two compounds with tubulin has been studied in detail in our laboratory (Leynadier *et al.*, 1993). The R- and S-isomers have the same fluorescence properties in solution but differ drastically after binding to tubulin. In presence of tubulin, the R-isomer showed a 60−70-fold increase in the fluorescence intensity of the R-tubulin complex with respect to free ligand, whereas the S-isomer showed only a 4-fold increase. The binding of the R- and S-isomers implicated tryptophanyl residues, quenched the intrinsic tubulin fluorescence, and increased the ligand fluorescence signal by energy transfer. They bound to or near the tubulin colchicine 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 R and S, respectively, and to several other low-affinity binding sites (Leynadier *et al.*, 1993). Displacement experiments with podophyllotoxin, a colchicine ring A analog, showed that R- and S-isomer binding implicated the ring A locus (see Chart 1). In these experiments, using a blocking agent like TME had not clearly established that the colchicine ring C was implicated. The R- and S-isomers

* To whom correspondence should be addressed.

[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1995.

[1] Abbreviations: NSC 613863 (*R*)-(+) and NSC 613862 (*S*)-(−) (CI980), ethyl 5-amino-2-methyl-1,2-dihydro-3-phenylpyrido[3,4-*b*]-pyrazin-7-yl carbamate; C179, ethyl 5-amino-2-methyl-3-phenylpyrido-[3,4-*b*]pyrazin-7-yl carbamate; NSC 337238, ethyl 5-amino-3-phenylpyrido[3,4-*b*]pyrazin-7-yl carbamate; NSC 330770, ethyl 5-amino-1,2-dihydro-3-phenylpyrido[3,4-*b*]pyrazin-7-yl carbamate; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; TME, tropolone methyl ether; TMB, 2,3,4,4′-tetramethoxy-1-1′-biphenyl-4-carboxylic acid; TKB, 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl; TBO, 2,3,4-trimethoxy-1,1′-biphenyl-4′-ol.

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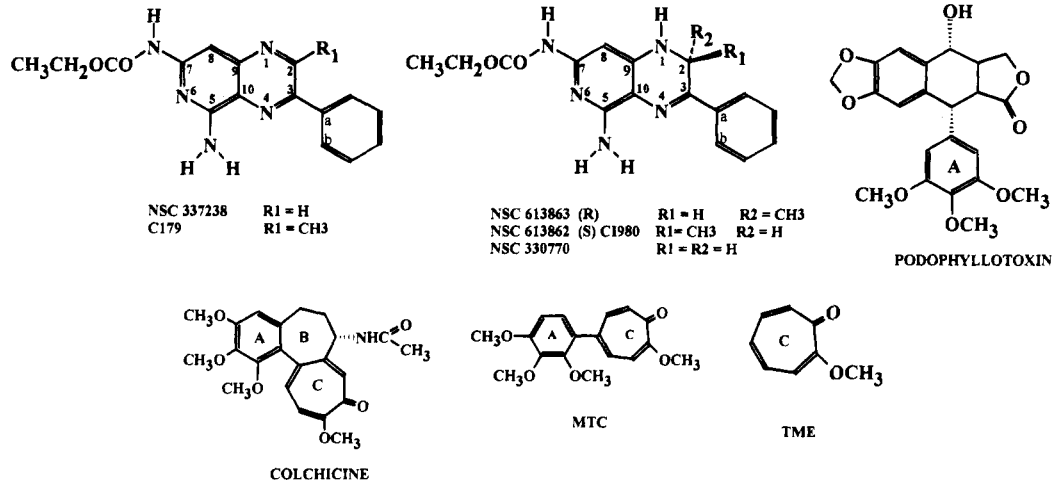
Chart 1



inhibited the polymerization of microtubules *in vitro*. The half-inhibitory concentrations are 0.4−0.7 μM for the S-isomer and 1.6−2.1 μM for the R-isomer (De Ines *et al.*, 1994). Like colchicine, both enantiomers were shown to induce GTPase activity (Leynadier *et al.*, 1993) and the formation of abnormal polymers. Cellular studies showed that the S-enantiomer is the more potent cellular microtubule and mitotic inhibitor (De Ines *et al.*, 1994).

The R- and S-isomers differ essentially by the orientation of the methyl group in C_2, i.e., foreground for the S-isomer and background for the R-isomer. To further study the mechanism of binding of these compounds, we studied three analogs (see Chart 1). C179 (Temple *et al.*, 1983) and NSC 337238 (Temple *et al.*, 1983) were tested with two goals in mind. The first was to check the interaction of tubulin with an achiral compound (C179) that has the methyl group in the molecular plan. The second was to examine the binding of tubulin with an analog that has no methyl group in C_2 (NSC 337238). However, the double bond between N_1 and C_2 makes C179 and NSC 337238 more conjugated and planar than the R- and S-isomers. Furthermore, our studies with R- and S-isomers indicated that the flexibility of the 1,2-dihydropyrido[3,4-*b*]pyrazine ring and the internal rotation of substituents play a major role in the differences elicited by the interaction with tubulin (Leynadier *et al.*, 1993). Consequently, to examine these observations, we studied NSC 330770 (Temple *et al.*, 1982) to elucidate the differences between R- and S-isomers when they are liganded to tubulin.

This report describes the inhibition of polymerization, the binding, the localization of the interaction, and the effects on the conformational parameters of tubulin for C179, NSC 337238, and NSC 330770. The results show that C179 interacted poorly with tubulin and that NSC 330770 was a powerful inhibitor that bound strongly to tubulin. NSC 337238 had an intermediate behavior. CNDO/2 calculations of the R- and S-isomers, C179, NSC 337238, and NSC 330770 specified the molecular mechanism of the interaction. Finally, we compared the binding of the R- and S-isomers with that of their analogs in order to further characterize the ethyl 5-amino-3-phenylpyrido[3,4-*b*]pyrazin-7-yl binding site of tubulin.

MATERIALS AND METHODS

Tubulin. Tubulin was purified from calf brains by ammonium sulfate fractionation and ion exchange chromatography. The protein was stored in liquid nitrogen and prepared as described (Weisenberg *et al.*, 1968; Lee *et al.*, 1973; Andreu *et al.*, 1984). Protein concentrations were determined spectrophotometrically with an extinction coefficient of $\epsilon_{275\ nm}$ = 1.07 L g^{-1} cm^{-1} in 0.5% SDS in neutral aqueous buffer or with $\epsilon_{275\ nm}$ = 1.09 L g^{-1} cm^{-1} in 6 M guanidine hydrochloride. The tubulin-colchicine complex was prepared as described (Andreu *et al.*, 1982a); its concentration was determined spectrometrically at $\epsilon_{275\ nm}$ = 1.14 L g^{-1} cm^{-1} in 1% SDS in aqueous buffer (Andreu *et al.*, 1982a). All experiments were carried out in PG buffer (10 mM sodium phosphate, 0.1 mM GTP, pH 7).

Chemicals. Colchicine and podophyllotoxin were from Aldrich Chemical Co. Extinction coefficients of $\epsilon_{290\ nm}$ = 3700 M^{-1} cm^{-1} for podophyllotoxin and $\epsilon_{352\ nm}$ = 14 200 M^{-1} cm^{-1} for colchicine were used for the spectrophotometric measurements of their concentrations. MTC [2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one] and TME (tropolone methyl ether) were a gift from Dr. Fitzgerald. Their concentrations were measured spectrophotometrically with extinction coefficients of $\epsilon_{343\ nm}$ = 17 600 M^{-1} cm^{-1} for MTC (Lee *et al.*, 1973) and $\epsilon_{345\ nm}$ = 6960 M^{-1} cm^{-1} for TME (Andreu *et al.*, 1984). NSC 613863 (*R*)-(+) (Temple & Rener, 1989), NSC 613862 (*S*)-(−) (CI980) (Temple & Rener, 1989), and their structural analogs NSC 330770 (Temple *et al.*, 1982) and NSC 337238 (Temple *et al.*, 1983) were synthesized as described by Temple *et al.* (1982, 1983). C179 was prepared as described in Temple *et al.* (1983) but required repeated treatment with potassium permanganate. Stock solutions were made in Me_2SO and kept at −20 °C. Their concentrations were determined spectrometrically with extinction coefficients of 15 400 and 15 100 M^{-1} cm^{-1} at 374 nm for the R- and S-isomers, respectively. For NSC 330770, NSC 337238, and C179, the extinction coefficients, ϵ, were determined by dissolving dry crystals of the compounds in Me_2SO and then diluting the solution with 10 mM sodium phosphate buffer, pH 7. The UV−visible spectra were then recorded. Three independent determinations gave $\epsilon_{374\ nm}$ = 12 800 ± 430 M^{-1} cm^{-1} for NSC 330770 and $\epsilon_{281\ nm}$ = 28 200 M^{-1} cm^{-1} for C179. Given the low solubility of NSC 337238 in aqueous buffer, we added Me_2SO up to 5% in the phosphate buffer. The extinction coefficient of NSC 337238, found by the method described above, was $\epsilon_{410\ nm}$ = 6700 ± 143 M^{-1} cm^{-1}. All the experiments with this compound were done with 5% Me_2SO except for polymerization and GTPase assays.

Ethylene glycol and GTP disodium salt were from Fluka. Glycerol, $MgCl_2$, Me_2SO, and EGTA were from Sigma [γ-^{32}P]GTP triethylammonium salt (10 mCi/mmol) was from Amersham International. All other chemicals were of reagent grade.

Binding Measurement by Fluorimetric Titration. Fluorescence measurements and uncorrected spectra were obtained with a Perkin-Elmer Luminescence Spectrometer 50 with slit widths of 5/5 nm monitored by an IBM PS2 computer and also with a Kontron SFM 25 spectrofluorimeter with slit widths of 5/5 nm. Fluorescence spectra were obtained in PG buffer, pH 7, by using 0.2 (excitation direction) × 1 cm cells (Hellma) thermostated at the desired temperature by circulating water from an external water bath.

Fluorescence Ligand Titrations. When NSC 330770 and C179 were excited at 380 nm and NSC 337238 was excited at 410 nm in presence of tubulin, an increase in fluorescence intensity and a blue shift of emission maxima were observed. Tubulin and tubulin−colchicine complex (1−3 μM) were titrated with various total NSC compounds at concentrations giving no appreciable inner filter effect (OD < 0.05). The fluorescence intensities obtained at maximum emission wavelengths (460 nm for NSC 330770 and 490 nm for NSC 337238 and C179) were corrected for the effects of low-affinity binding sites by subtracting the fluorescence intensities of the tubulin−colchicine−ligand complex from those of the protein−ligand, as described by Leynadier *et al.* (1993) for the R- and S-isomers. Then the fluorescence-corrected data (F_{corr}) were fitted to the saturation curve equation by means of nonlinear least-squares regression analysis.

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

F_{max} is the plateau fluorescence value. Concentrations (bound ligand $[L_b]$ and free ligand $[L_f]$) and binding parameters (the apparent stoichiometry n and the dissociation constant K_d) were determined with the equation

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

where $[L_0]$ and $[P_0]$ are the total ligand and protein concentrations, respectively. The algorithm starts with an arbitrary opening set of n and K_d values. With these values, $[L_b]$ and $[L_f]$ are calculated and then the nonlinear least-squares regression analyses are executed. The initial set is corrected in the next step by a Newton−Gauss procedure. This iterative procedure is continued until the minimum sum of squared deviations between experimental and calculated values of F_{corr} is obtained.

Fluorescence Protein Quenching Titrations. Quenching of the intrinsic protein fluorescence signal by ligands was also employed to estimate the binding parameters. Tubulin (1−3 μM) was titrated by various concentrations of NSC compounds. The fluorescence measurements were performed with an excitation wavelength of 295 nm in order to specifically excite the tubulin tryptophanyl residues. The emission wavelength was fixed at 340 nm. The inner filter effects were corrected according to Lakowicz (1983) as follows:

$$F_{corr} = F_{obs} \exp[(A_{exc} + A_{em})/2]$$

F_{obs} and F_{corr} are the observed and corrected fluorescence values at the emission wavelengths. A_{exc} and A_{em} are the absorptions at the excitation and emission wavelengths, respectively, calculated with $A_x = \epsilon_x l_x C$, in which x is the excitation or emission direction, ϵ is the extinction coefficient, l is the path length of the cell in the excitation and emission directions, and C is the ligand concentration. The corrected fluorescence titration curves were inverted and fitted by using the nonlinear least-squares regression analysis described above.

Miscellaneous Procedures: Polymerization. Microtubule assembly was performed in 10 mM sodium phosphate buffer, 1 mM EGTA, 8 mM $MgCl_2$, 3.4 M glycerol, pH 6.7. The reaction was started by warming the samples to 37 °C in thermostated cuvettes (1 × 0.2 cm), and the mass of polymer formed was monitored by turbidimetry at 350 nm with Beckman DU70 and DU7400 spectrophotometers. Samples containing NSC compounds and their controls had less than 2% residual Me_2SO.

GTPase Assay. The tubulin was freed of the colchicine-independent contaminant GTPase activity found in tubulin preparations by passing it through a Sephacryl S-300 HR (Andreu & Timasheff, 1981). The ligand-induced GTPase activity was assayed as described in Peyrot *et al.* (1992). The protein samples (1−2 mg/mL) and blanks were preincubated for 30 min at 0 °C in 10 mM sodium phosphate, 1 mM $MgCl_2$ buffer, pH 7, containing 10^6 dpm of [γ-^{32}P]GTP at a final concentration of 0.1 mM GTP in order to allow isotopic equilibration. The NSC compounds, colchicine, and podophyllotoxin were added at saturating concentration in 0.15 mL aliquots of the samples. The enzyme reaction was started by placing the tubes in a 37 °C water bath and stopped by adding 0.450 mL of 0.25 N perchloric acid. The phosphomolybdate extraction procedure of Nishizuka *et al.* (1968) was then carried out. A 1 mL aliquot of the organic phase was removed and mixed with 10 mL of Bray's solution and then counted in a Beckman LS-1701 liquid scintillation counter.

Formation of Abnormal Polymers. The assembly reaction of tubulin into abnormal polymers which was induced by NSC 330770 and NSC 337238 in 10 mM sodium phosphate buffer, 16 mM $MgCl_2$, 0.1 mM GTP, pH 7, at 37 °C was monitored by turbidimetry at 475 nm in order to minimize light absorption by compounds. Tubulin (0.5−4 mg mL^{-1}) was mixed with a saturating concentration of NSC compounds in thermostated cuvettes (1 × 0.2 cm). The residual quantity of Me_2SO was less than 2%.

Competition Experiments. Competition experiments were done with thermostated cells (1 × 1 cm) at 25 °C and a Kontron SFM25 spectrofluorimeter. The excitations were conducted at wavelengths chosen to minimize the inner filter effect due to light absorption by the products. Data were acquired directly by a personal computer at a rate of 1 point per second. Competition experiments between the R-isomer and NSC 330770 or NSC 337238 were carried out at an excitation wavelength of 380 nm, and the data were collected at an emission wavelength of 460 nm. Competition experiments between NSC 330770 and MTC and podophyllotoxin were done with the above conditions. Excitation and emission wavelengths for competition experiments between NSC 337238 or C179 and MTC and podophyllotoxin were 410 and 490 nm, respectively. To suppress the inner filter effect, we did the experiments with TME at an excitation wavelength of 420 nm and an emission wavelength of 460

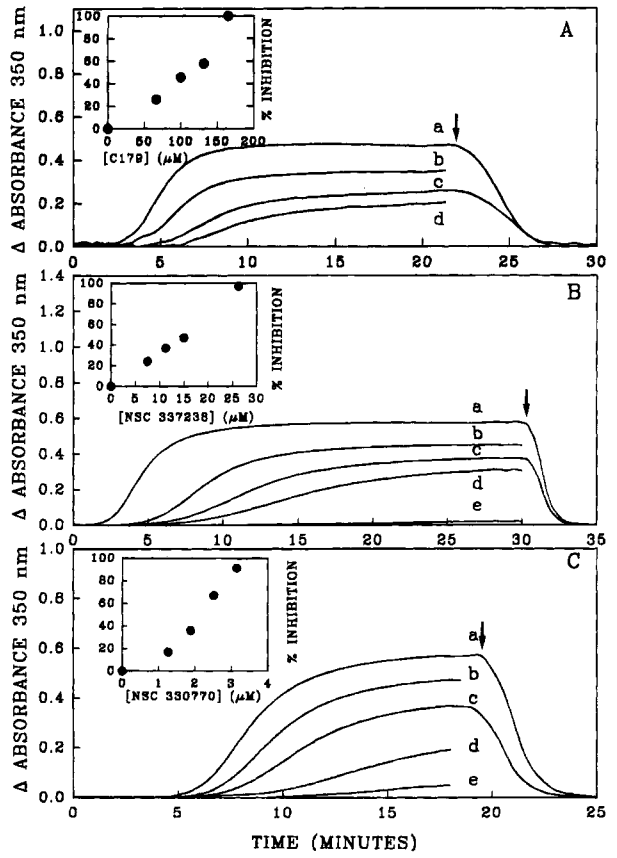


FIGURE 1: Effect of C179, NSC 337238, and NSC 330770 on the turbidity time course of *in vitro* microtubule assembly. The reaction was started by warming the solution from 4 to 37 °C. Panel A shows (a) tubulin at 1.6 mg mL^{-1} and (b–d) aliquots of the same solution with 66×10^{-6}, 100×10^{-6}, and 132×10^{-6} M C179. Panel B shows (a) tubulin at 2.3 mg mL^{-1} and (b–e) aliquots of the same solution with 7.5×10^{-6}, 11.3×10^{-6}, 15.0×10^{-6}, and 26.3×10^{-6} M NSC 337238. Panel C shows (a) tubulin at 2.1 mg mL^{-1} and (b–e) aliquots of the same solution with 1.26×10^{-6}, 1.89×10^{-6}, 2.52×10^{-6}, and 3.16×10^{-6} M NSC 330770. At the time indicated by the arrow, the samples were cooled to 10 °C. The insets show the percentage of turbidity inhibition as a function of total ligand concentrations.

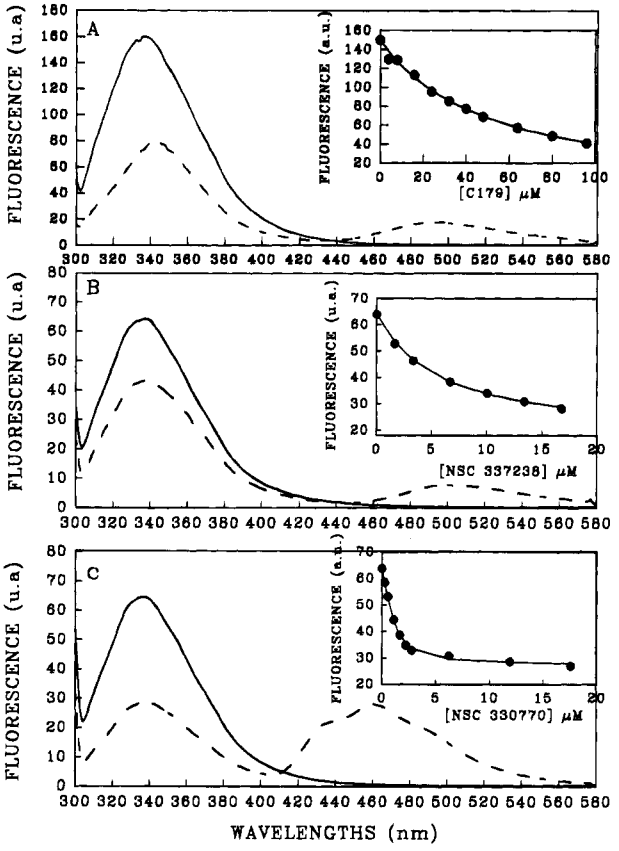


FIGURE 2: (A) Fluorescence changes in the protein emission spectra induced by the tubulin–C179 interaction ($\lambda_{exc} = 295$ nm). Tubulin (5 μM) in PG buffer, pH 7, was mixed with no (—) or 32 μM C179 (- - -). The inset shows the protein-quenching titration curve produced by C179 binding to 5 μM tubulin at 25 °C; the solid line is the fitting curve obtained as described in Materials and Methods. (B) Fluorescence changes in the protein emission spectra induced by the tubulin–NSC 337238 interaction ($\lambda_{exc} = 295$ nm). Tubulin (1.3 μM) in PG buffer, 5% Me_2SO, pH 7, was mixed with no (—) or 3.36 μM NSC 337238 (- - -). The inset shows the protein-quenching titration curve produced by NSC 337238 binding to 1.3 μM tubulin at 25 °C; the solid line is the fitting curve obtained as described in Materials and Methods. (C) Fluorescence changes in protein emission spectra induced by the tubulin–NSC 330770 interaction ($\lambda_{exc} = 295$ nm). Tubulin (1.3 μM) in PG buffer, pH 7, was mixed with no (—) or 3.64 μM NSC 330770 (- - -). The inset shows the protein-quenching titration curve produced by NSC 330770 binding to 1.3 μM tubulin at 25 °C. Symbols represent the experimental points (collected at 340 nm), and the solid line is the fitting curve obtained as described in Materials and Methods.

nm, except for NSC 337238, for which the emission wavelength was 490 nm.

Theoretical Chemistry Calculation. For molecular orbital calculations, the CNDO/2 method was used with the original parameters of Pople and Segal (1966). The calculations served to determine the dipole moments, the energy of HOMO (highest occupied molecular orbital) and of LUMO (lowest unoccupied molecular orbital) and the π-electron density contribution from each participant atoms in these orbitals (Loew *et al.*, 1984). In the absence of X-ray data, geometries were obtained by using the usual angles and bond lengths (Sutton, 1958) and then by refining them with a personal computer program.

RESULTS

Effects on Purified Tubulin Assembly in Vitro. Figure 1A–C show the effects of C179, NSC 337238, and NSC 330770 on the turbidimetry time course of microtubule assembly. A clear inhibition was noted, and the rate of assembly as well as the final amount of microtubules was lower in the presence of the three drugs. The turbidity generated by the self-assembly of 1.6×10^{-5} M pure tubulin (see inset of Figure 1A) was reduced by 50% by 100 μM C179. The concentration of NSC 337238 needed to halve the turbidity generated by the self-assembly of 2.3×10^{-5} M tubulin was 14 μM (see inset Figure 1B). For NSC 330770 (inset Figure 1C), 2 μM halved the turbidity generated by assembly of 2.1×10^{-5} M tubulin. In the presence of all three drugs, when the samples were cooled to 10 °C, the polymers depolymerized as well as the control assembled without drugs.

These results indicated that the inhibition of microtubule assembly by C179 was stoichiometric, while that by NSC 337238 and NSC 330770 was substoichiometric. C179, however, displayed a weak inhibition, indicating that this compound may weakly bind to tubulin.

Determination of the Interaction of C179, NSC 337238, and NSC 330770 with Tubulin by Fluorescence Spectroscopy: Fluorescence Quenching of Tubulin by C179, NSC 337238, and NSC 330770. Examination of the fluorescence of tubulin in presence of these compounds (Figure 2A–C)

revealed a decrease in the intrinsic protein fluorescence signal at 340 nm and an increase in fluorescence intensity at 490 nm for C179 and NSC 337238 and at 460 nm for NSC 330770. An isobestic point at 440, 444, and 411 nm was observed for C179, NSC 337238, and NSC 330770, respectively. For NSC 337238, the shapes of spectra generated by the interaction with tubulin were similar to those of the C179−tubulin interaction. The insets of Figure 2A,B show typical quenching titration curves for the association of C179 and NSC 337238. It was difficult to accurately determine the binding stoichiometry of these two ligands. Thus, for C179 and NSC 337238, the number of sites (n) was set to 1 and the apparent affinity constants were estimated to be (2.41 ± 0.22) × 10^4 M^{-1} and (2.37 ± 0.42) × 10^5 M^{-1}, respectively. The inset of Figure 2C shows the tubulin-quenching titration curve of NSC 330770 binding. The compound bound to one site (n = 1.00 ± 0.14) on tubulin with an apparent affinity constant K_a = (3.69 ± 0.96) × 10^6 M^{-1}. All the values are the means of three determinations from three tubulin preparations.

Ligand Fluorescence Titrations of Tubulin. The binding of the ligands to tubulin was also examined with the ligand fluorescence signal. As shown in Figure 3A−C, C179, NSC 337238, and NSC 330770 had a weak fluorescence signal in neutral aqueous solution. C179 had a fluorescence excitation maximum at 380 nm and a maximum of emission at 520 nm (Figure 3A). NSC 337238 had a fluorescence excitation maximum at 410 nm and an emission maximum at 520 nm (Figure 3B). Finally, the NSC 330770 fluorescence spectrum had a maximum of excitation at 380 nm and a maximum of emission at 488 nm (Figure 3C). As expected, the ligand fluorescence signals increased and a blue shift appeared upon binding to tubulin; the spectra generated by the interaction of tubulin with C179, NSC 337238, and NSC 330770 were characterized by a maximum emission at 490 nm for C179 and NSC 337238 and at 460 nm for NSC 330770. Compared with free ligand fluorescence, those of C179 and NSC 337238 were increased 3−4-fold and that of NSC 330770 was increased 6-fold (Figure 3A−C). Titration of tubulin with various concentrations of C179, NSC 337238, and NSC 330770 showed a nonsaturated curve due to the presence of low-affinity binding sites. The titration curve was corrected and analyzed as described in Materials and Methods. As for protein quenching titrations, for C179 and NSC 337238 the stoichiometry was set to 1 and equilibrium constants of K_a = (2.17 ± 0.33) × 10^4 M^{-1} and K_a = (2.86 ± 0.29) × 10^5 M^{-1} were obtained. NSC 330770 bound to 0.96 ± 0.01 site with an affinity constant of K_a = (2.9 ± 0.34) × 10^6 M^{-1}. All of these values are in agreement with those determined by fluorescence quenching of tubulin.

Determination of the Thermodynamic Parameters of the Interaction of the R- and S-isomers, C179, NSC 337238, and NSC 330770 with Tubulin. The estimations of the apparent binding affinity constants of the R- and S-isomers, C179, NSC 337238, and NSC 330770 by ligand fluorescence titration at different temperatures were used to determine the standard enthalpy changes of the reaction ($\Delta H°$) by Van' t Hoff plots. The standard entropy variations ($\Delta S°$) were calculated by the following equations:

$$\Delta G° = \Delta H° - T\Delta S° \quad \text{and} \quad \Delta G° = -RT \ln K_a$$

The thermodynamic parameters are summarized in Table 1.

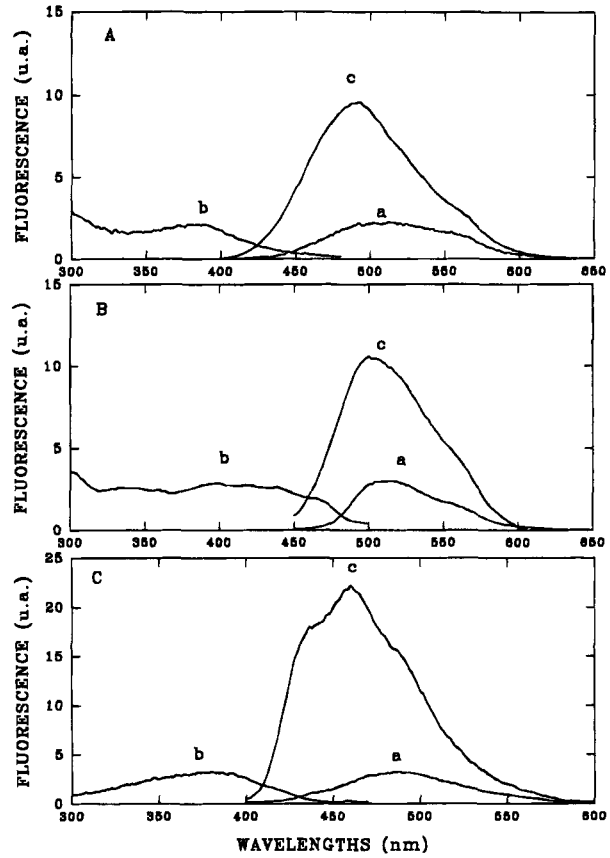


FIGURE 3: Fluorescence changes produced by the interaction of C179, NSC 337238, and NSC 330770 with tubulin. (A) C179 fluorescence excitation and emission spectra: (a) uncorrected emission spectrum of 48 μM C179 in PG buffer (excitation at 380 nm); (b) uncorrected excitation spectrum of 48 μM C179 (emission at 520 nm); (c) emission spectrum of the interaction of 48 μM C179 with 5 μM tubulin (excitation at 380 nm). (B) NSC 337238 fluorescence excitation and emission spectra: (a) uncorrected emission spectrum of 25 μM NSC 337238 in PG buffer, 5% Me_2-SO (excitation at 410 nm); (b) uncorrected excitation spectrum of 25 μM NSC 337238 (emission at 520 nm); (c) emission spectrum of the interaction of 25 μM NSC 337238 with 5 μM tubulin (excitation at 410 nm). (C) NSC 330770 fluorescence excitation and emission spectra: (a) uncorrected emission spectrum of 3.4 μM NSC 330770 in PG buffer (excitation at 380 nm); (b) uncorrected excitation spectrum of 3.4 μM NSC 330770 (emission at 488 nm); (c) emission spectrum of the interaction of 3.4 μM NSC 330770 with 1.2 μM tubulin (excitation at 380 nm).

Table 1: Thermodynamic Parameters of the Binding of the R- and S-Isomers and Structural Analogs C179, NSC 337238, and NSC 330770 to Tubulin at 25 °C

	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J mol^{-1} K^{-1})
R	−37.4 ± 2.1	−42 ± 7	−15
S	−37.7 ± 3.3	−87 ± 18	−165
C179	−24.6 ± 2.7	−29 ± 2	+15
NSC 337238	−31.1 ± 2.7	−33 ± 5	+6
NSC 330770	−36.9 ± 4.3	−57 ± 8	−67

The R- and S-isomers and NSC 330770 had large exothermic standard enthalpy changes and negative entropy changes. C179 and NSC 337238 also had an exothermic standard enthalpy changes, but they had positive entropy changes.

Specificity and Localization of the Interaction. The two enantiomers, R and S, bind to tubulin at the same site that seems to overlap the well-known colchicine site (see introduction). To check whether structural analogs used the same binding site, we performed competition experiments. Because of the weak equilibrium constant of C179, the

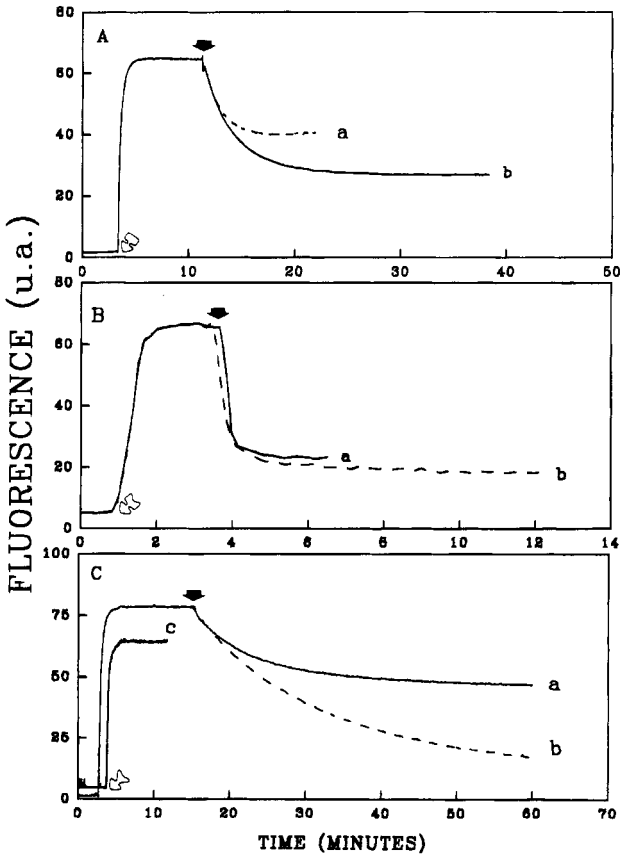


FIGURE 4: (A) Time course of the fluorescence change upon binding of 2.5 μM R-isomer (open arrow) to 2.5 μM tubulin at 25 °C. At the times indicated by the black arrow, 25 μM (each) NSC 330770 (trace a) and NSC 337238 (trace b) were added. (B) Time course of the fluorescence change upon binding of 2 μM NSC 337238 (open arrow) to 2 μM tubulin at 25 °C. At the times indicated by the black arrow, 92 μM MTC (trace a) and 400 μM podophyllotoxin (trace b) were added. (C) Time course of the fluorescence change upon binding of 2 μM NSC 330770 (open arrow) to 1.7 μM tubulin at 25 °C. At the times indicated by the black arrow, 92 μM MTC (trace a) and 400 μM podophyllotoxin (trace b) were added. Trace c shows the interaction of 2 μM NSC 330770 with 1.7 μM tubulin preincubated with 10 mM TME for 30 min at 25 °C.

concentrations necessary to displace R, S, NSC 330770, or NSC 337238 bound to tubulin were too high, hence these experiments were not done with C179. Figure 4A shows the fluorescence signal increase induced by the association of 2.25 μM R and 2.30 μM tubulin. Adding 25 μM NSC 337238 (trace a) at the equilibrium state of binding produced a decrease of only 40% in 10 min, whereas adding 25 μM NSC 330770 (trace b) induced a decrease of 60% in 20 min. Residual signals obtained in these displacement experiments were probably the consequence of a fast reassociation of the R-isomer with tubulin, or/and the contribution of the fluorescence of free and bound ligands. Finally, we sought to determine whether the structural analogs of colchicine were able to displace the binding of such molecules. NSC 337238 (Figure 4B) and NSC 330770 (Figure 4C) were displaced by podophyllotoxin (ring A colchicine analog) and MTC (ring A and C colchicine analog), as for the R- and S-isomers. The fast displacement of NSC 337238 produced by podophyllotoxin and MTC resulted from the lower-affinity binding constant of NSC 337238 for tubulin relative to those of the two colchicine analogs. Fast displacement was also observed with C179 (not shown). TME is a ring C colchicine analog with slow association and dissociation rate constants (Andreu *et al.*, 1984; Engelborghs *et al.*, 1992). It was thus necessary to preincubate tubulin with an excess of TME for 25 min at 25 °C to reach the equilibrium state of the formation of the complex. Figure 4C (trace c) shows that the amplitude of the fluorescence signal of NSC 330770 binding in presence of 10 mM TME decreased by 20−30%. Similar results were obtained for S, R, and NSC 337238. Competition experiments indicated that these compounds bound to tubulin at the same site involving the ring A and C loci of colchicine.

Conformational Effects of Binding of C179, NSC 337238, and NSC 330770 to Tubulin. The binding of colchicine, or its analog, MTC, to tubulin has several consequences: inhibition of microtubule formation, small changes in the far-ultraviolet protein circular dichroism spectrum, appearance of GTPase activity, and tubulin polymerization into structures that geometrically differ from microtubules but have the same behavior (Andreu & Timasheff, 1982b; Saltarelli & Pantaloni, 1982; Andreu *et al.*, 1983). The last two properties were examined in tubulin liganded to C179, NSC 337238, and NSC 330770.

GTPase Activity. The GTPase activity induced in tubulin by these compounds was measured with colchicine (100%) and podophyllotoxin (0%) as references. The protein freed of contaminant enzyme activity (see Materials and Methods) showed no GTPase activity for C179 and NSC 337238, whereas 24% ± 10% of the activity of the tubulin-colchicine complex was detected for NSC 330770.

Induction of Abnormal Polymer Formation. In glycerol-free buffer, tubulin did not form microtubules (see Figure 5A, dashed line). The formation of anomalous tubulin polymers with a critical concentration of ca. 1 mg mL^{-1} upon stoichiometric binding of the drug to α-β tubulin is characteristic of colchicine (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982b; Andreu *et al.*, 1983), MTC (Andreu *et al.* 1984), allocolchicine, and TCB (Medrano *et al.*, 1989). About 10^{-3} M C179 was needed to saturate 95% of the tubulin (2 mg mL^{-1}). In this concentration range, the compound was not fully soluble, thus experiments on induction of abnormal polymers were not performed with C179. Figure 5 shows the increase in turbidity induced by the interaction of NSC 337238 (Figure 5A) and NSC 330770 (Figure 5B) with tubulin. The polymerization was reversed by cooling the samples to 10 °C. The polymerization induced by an excess of NSC 337238 (6.2×10^{-5} M) was characterized by a lag time and a critical concentration Cr = 2.1 mg mL^{-1} (see inset of Figure 5A), which is characteristic of nucleated cooperative self-assembly (Oosawa & Asakura, 1975). An excess of NSC 330770 (8.2×10^{-5} M) over tubulin also produced a reversible turbidity increase but with a lower critical concentration Cr = 0.53 mg mL^{-1} (inset of Figure 5B). On electron microscopy, negatively stained, fixed preparations showed the absence of microtubules and the formation of large, filamentous structures. These characteristics are very similar to those of the polymerization of the (*R*)- and (*S*)-tubulin complexes (De Ines *et al.*, 1994).

Theoretical Chemistry Calculation. Analysis of the structure of our molecules by CNDO/2 molecular orbital methods provided more information about chemical characteristics:

(1) The determination of the dihedral angle between the pyridopyrazine ring and the phenyl group (i.e., $N_4-C_3-C_a-C_b$) (see Chart 1) gave the values ϕ = 10, 22, and 13°, for the R- and S-isomers and NSC 330770 and ϕ = 18 and 28° for NSC 337238 and C179, respectively.

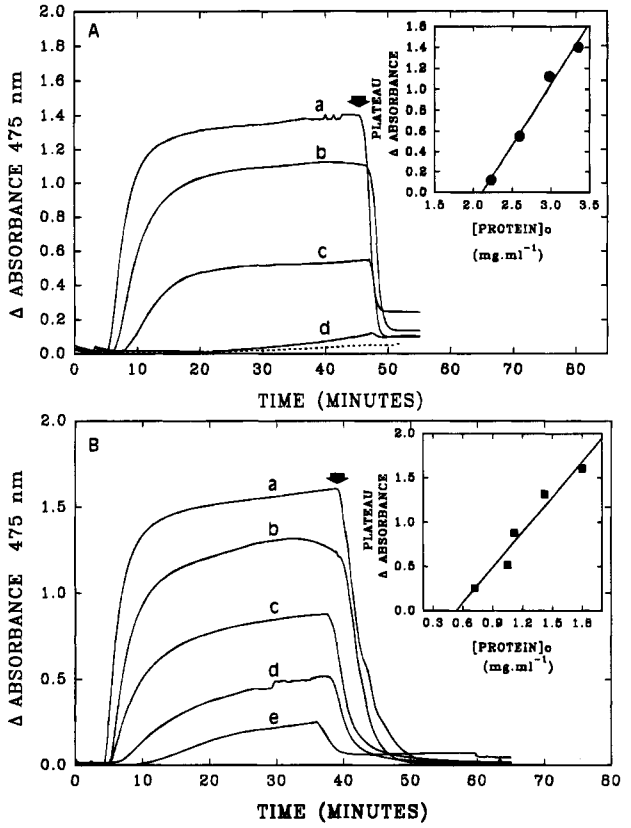


FIGURE 5: Effect of NSC 337238 and NSC 330770 on the formation of abnormal polymers in vitro. The reaction was started by warming the solutions from 10 to 37 °C. The arrow indicates when the samples were cooled to 10 °C. Panel A, 61 μM NSC 337238 with tubulin at 3.35 (a), 2.98 (b), 2.61 (c), and 2.23 mg mL^{-1} (d). Dashed line, tubulin at 1.86 mg mL^{-1} without drug. Panel B, 82 μM NSC 330770 with tubulin at 1.86 (a), 1.49 (b), 1.12 (c), 1.05 (d), and 0.74 mg mL^{-1} (e). The insets show dependence of polymer formation versus total tubulin concentration.

(2) The energy of the HOMO, i.e., the energy required to remove an electron from the highest filled orbital was smaller for the R- and S-isomers and NSC 330770 than for the other compounds. This indicates that these molecules may be good electron donor molecules (see Table 2). Moreover, the π-electron densities in the HOMO revealed that, in this molecular orbital, the participation of the atomic orbitals of atoms N_4, C_3, and C_{10} was large for the R- and S-isomers and NSC 330770 whereas for NSC 337238 and C179 the electron density was preferentially localized in N_4 and N_1 (see Table 2).

(3) The energy of the LUMO corresponding to the energy gain brought by an electron that then must occupy the lowest unoccupied orbital was higher for C179 and NSC 337238 than for the R- and S-isomers and NSC 330770 (Table 2). This indicates that the former two compounds possessed a good electron affinity.

(4) The dipole moment is an important parameter which accounts for the biochemical activity. It was found to be 1.79, 2.88, 1.77, 0.21, and 0.48 debyes for R, S, NSC 330770, NSC 337238, and C179, respectively (Table 2).

DISCUSSION

Interaction of R, S, C179, NSC 337238 and NSC 330770 with Tubulin and Microtubules: Binding to Tubulin. The binding of C179, NSC 337238, and NSC 330770 quenched the intrinsic tubulin fluorescence and increased the ligand fluorescence signal, both of which suggest that the tryptophanyl residues are close enough to permit an energy transfer. Similar results have been reported for colchicine (Andreu *et al.*, 1982a) and the R- and S-isomers (see introduction). C179, the methylated dehydrogenated achiral R,S-analog, bound to tubulin with a weak apparent association constant whereas the substitution of -CH_3 by -H on C179 led to a 10-fold increase in the equilibrium affinity constant (see Table 1). NSC 330770, the demethylated hydrogenated achiral R,S-analog, bound to tubulin with an affinity constant close to that of the R-isomer. The inhibition of R-isomer binding by an excess of NSC 337238 or NSC 330770 showed that these compounds bind to tubulin at the same site. MTC, podophyllotoxin, and TME inhibited the binding of R, S, NSC 337238, and NSC 330770. It thus appears that the binding of such molecules to tubulin implicates the ring A and C loci.

The binding thermodynamic parameters (Table 1) showed a large exothermic enthalpy change and a large negative entropy variation for the most active compounds (R, S, and NSC 330770). The large negative enthalpy change suggested that the interactions are dominated by electrostatic rather than by hydrophobic interaction. The high entropy variation values for the S-isomer and the NSC 330770 indicated that large structural conformation change occurred upon their binding with tubulin, as shown by their ability to induce the formation of anomalous structures. These observations agree with our results obtained in stopped-flow kinetics studies (P. Barbier, V. Peyrot, C. Dumortier, A. D'Hoore, G. A. Rener, and Y. Engelborghs, paper in preparation). It should be noted that only the thermodynamic parameters ($\Delta H°$ and $\Delta S°$) and critical concentration values inducing the formation of abnormal polymers made it possible to clearly differentiate the behaviors of NSC 330770 and R-isomer. For C179 and NSC 337238, the negative enthalpy and the low positive entropy changes indicate that less conformational restructuration occurred in the complex.

Inhibition of Microtubule Formation. First, all of these compounds inhibited *in vitro* microtubule assembly in substoichiometric mode, except for C179, but the most potent inhibitor remained the S-isomer (50% of polymerization inhibition was obtained with 0.4−0.7 μM). A 4-fold higher concentration of the R-isomer or its achiral analog NSC 330770 was necessary to obtain such inhibition.

Induction of Abnormal Polymerization. Induction of abnormal polymerization is a characteristic of colchicine ligands, such as MTC, allocolchicine, and biphenyl analogs (Andreu *et al.*, 1983, 1984, 1991; Medrano *et al.*, 1989). It has been proposed that the formation of abnormal structures was related to the ability to anchor the ligand in proper orientation in the colchicine ring C binding site in tubulin (Andreu *et al.*, 1991). Furthermore, the R- and S-isomers act in the same manner as colchicine, by inducing formation of such structures. As expected, the present study showed that binding of stoichiometric concentrations of NSC 337238 and NSC 330770 to tubulin also induced the formation of anomalous polymers. NSC 330770, the S-isomer, and MTC (Andreu *et al.*, 1984) had the same critical protein concentration (Cr $\approx$ 0.5 mg mL^{-1}) whereas NSC 337238 had a 4-fold higher one (Cr = 2.1 mg mL^{-1}). The R-isomer and colchicine (Andreu *et al.*, 1983) had the same critical concentration ($\approx$1 mg mL^{-1}). All these molecules appear to bind to tubulin one the ring C colchine binding site and to perturb the protein−protein interactions. This was confirmed by the inhibition of their binding by TME, a

Table 2: Chemical Parameters of R, S, C179, NSC 337238, and NSC 330770 Determined by Theoretical Chemistry Calculation CNDO/2

	dihedral angle ϕ	energy of HOMO (eV)	energy of LUMO (eV)	dipole moment (debyes)
R-isomer	10°	−9.798 [N_4, 0.118; C_3, 0.169; C_{10}, 0.149][a]	2.026 [N_4, 0.150; C_3, 0.177][a]	1.79
S-isomer	22°	−9.624 [N_4, 0.116; C_3, 0.163; C_{10}, 0.154][a]	2.121 [N_4, 0.153; C_3, 0.178][a]	2.88
C179	28°	−10.261 [N_4, 0.178; N_1, 0.188][a]	1.734 [N_4, 0.207; N_1, 0.142; C_5, 0.167][a]	0.48
NSC 337238	18°	−10.373 [N_4, 0.180; N_1, 0.187]4a	1.679 [N_4, 0.207; N_1, 0.142; C_5, 0.168][a]	0.21
NSC 330770	13°	−9.839 [N_4, 0.110; C_3, 0.171; C_{10}, 0.152][a]	1.991 [N_4, 0.156; C_3, 0.176][a]	1.77

[a] Localization of the atomic orbitals implicated in HOMO and LUMO and the value for this probability.

monocyclic ring C analog of colchicine.

GTPase Activity. The protein conformational changes generated by the binding of colchicine and its analogs induce GTPase activity (David-Pfeuty *et al.*, 1979; Andreu *et al.*, 1984). Binding to both subsites A and C of colchicine seems to be required to provoke hydrolysis of GTP (Andreu *et al.*, 1991). Podophyllotoxin and MDL 27048 present no GTPase activity and bind to colchicine ring A subsite (Andreu & Timasheff, 1981; Peyrot *et al.*, 1992). The R- and S-isomers were found to elicit GTPase activity in tubulin (Leynadier *et al.*, 1993). C179 and NSC 337238 presented no activity, whereas NSC 330770 induced a GTPase activity similar to that found for the R-isomer. Moreover, the loss of ability of C179 and NSC 337238 to induce hydrolysis of GTP proves that they are not properly oriented in the C locus when they interact with tubulin.

For C179, NSC 337238, NSC 330770, and the two isomers, the apparent binding constant values are correlated with their ability to elicit GTPase activity. This suggests that a high-affinity binding constant and the 1,2-dihydro structure with the twist between the N_1 and C_2 atoms are necessary to produce GTPase activity.

It has been shown that the biphenyl colchine analog TMB, which induces GTPase activity, is not able to induce formation of anomalous structures (Andreu *et al.*, 1991). On the other hand, other biphenyl colchicine analogs like TKB and TBO, which both have the same GTPase activity, do not present the same behavior in the formation of abnormal polymers (Medrano *et al.*, 1989). Here, we also found it difficult to correlate formation of abnormal polymers and GTPase activity. Indeed, NSC 337238 did not induce GTPase activity but did generate the formation of abnormal polymers.

Theoretical Chemistry Calculations. The dipole moment of a molecule is an important parameter that accounts for its activity and is often used in studies of structure−activity relationships. Moreover, it reflects the presence of favorable orientation of electronegative substituents at certain key sites required for interaction. The R-isomer and NSC 330770 had the same dipole moment value and the same shape of bound fluorescence emission spectra (presence of three shoulders). These observations suggest that they link to tubulin in the same way. However, the fluorescence intensity increase developed by the interaction of NSC 330770 with tubulin was smaller than that of the R-isomer. This can be explained by a difference of three degrees in the dihedral angle between the 1,2-dihydropyrido[3,4-*b*]pyrazin ring and the phenyl group for these two compounds (see Table 2). The dihedral angle is larger for the S-isomer, C179, and NSC 337238, and these compounds show a slight increase in fluorescence intensity upon binding to tubulin (Leynadier *et al.*, 1993). These authors hypothesized that the drastic difference in fluorescence increase between the two isomers upon binding to tubulin resulted from a more hindered rotation of the phenyl group of the R-isomer, confining the R-isomer differently in its binding site, or from a different ionization state of the two molecules when they are bound to tubulin. Here, the first hypothesis seems to be confirmed, since the compounds with large dihedral angle values, i.e., C179, S-isomer, and NSC 337238, developed a weak increase in fluorescence intensity upon interaction with tubulin. This suggests that the phenyl group of these molecules is less anchored in the binding site than that of the R-isomer and NSC 330770. Moreover, Allam *et al.* (1995) reported that the Raman spectra of both isomers are modified upon binding to tubulin and that the Raman bands due to C=C stretching from the phenyl ring are more strongly modified for the R-isomer than for the S-isomer. They also concluded that both enantiomers differ by their orientation in front of the binding locus of tubulin.

The ionization potential (energy of the HOMO) and the electron affinity (energy of LUMO) were calculated. Results showed that the most effective compounds in the different biochemical tests used here are the best electron donors and the worst electron acceptors, i.e., the S-isomer, R-isomer, and NSC 330770 in decreasing order. Thus, a charge transfer must occur during the interaction between these molecules and tubulin. Many authors have implicated tryptophanyl residues in the tubulin colchicine-binding site (Andreu & Timasheff, 1982b; Maccioni & Seeds 1982; Lacey, 1988). These residues are normally regarded as good π-electron donors. Nevertheless, if an electron transfer occurred from tryptophan (donor) to the ligand (acceptor), then a decrease in the tryptophanyl fluorescence intensity and a red shift of their emission maximum should be observed (Slifkin, 1971); herein, only a quenching of the tryptophanyl fluorescence was observed. In the same way, if an electron transfer occurred from the ligand (donor) to the tryptophanyl residues (acceptor), then a decrease in the fluorescence intensity and a red shift should be observed; conversely, we observed a fluorescence intensity increase and a blue shift. We conclude that the electron transfer from these ligands to tubulin must

occur via other amino acid residues.

The electron density and the localization of the atomic orbitals probably implicated in the formation of the HOMO and LUMO showed that the electron density of C179 and NSC 337238 is preferentially distributed on atoms N_1 and N_4. On the other hand, for the two isomers and NSC 330770, the electron density is distributed on atoms C_3, N_4, and C_{10} and not on the C_2 atom responsible for chirality. Finally, it appears clearly that the position of the methyl group is not the crucial parameter for the activity; rather, the presence of the 1,2-dihydro structure, a large dipole moment, and an electron donor effect localized on atoms N_4, C_3, and C_{10} are necessary for an antitubulin agent with ethyl 5-amino-3-pyrido[3,4-*b*]pyrazine structure to be potent. Our results show that NSC 330770 is a powerful tubulin polymerization inhibitor and that it interacts strongly with tubulin. Furthermore, this compound appeared to be a potent inhibitor of the cytoplasmic microtubule polymerization of PtK2 cells as did the S-isomer (J. M. Andreu and C. De Ines, personal communication). In 1994, De Ines *et al.* demonstrated that cellular microtubule inhibition and mitotic arrest are strongly chiral-selective by a factor of ~20 in favor of the S-isomer. They also proposed several hypotheses to explain this difference, in particular, the presence of an additional, sensitive cellular target, different from tubulin, which would be chiral selective for the S-isomer. Now, the potent activity of NSC 330770, an achiral compound, allows us to rule out this hypothesis. Moreover, in 1989 Temple *et al.* reported that NSC 330770 inhibited the proliferation of cultured L1012 cells and showed antitumor activity against P388 leukemia in mice. Unfortunately, NSC 330770 has not been selected for clinical trials because it is not as stable as the S-isomer (CI980) and has a lower solubility, hampering its formulation for therapeutic administration.

ACKNOWLEDGMENT

We are grateful to Dr. Brouant of the Therapeutic Chemistry Laboratory (Faculté de pharmacie, Marseille) for the theoretical chemistry calculations. We thank Dr. T. J. Fitzgerald for the gift of MTC and TME and Drs. J. M. Andreu (C.I.B., Madrid) and C. Temple for comments on the manuscript.

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