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Roles of Ring C Oxygens in the Binding of Colchicine to Tubulin[†,‡]

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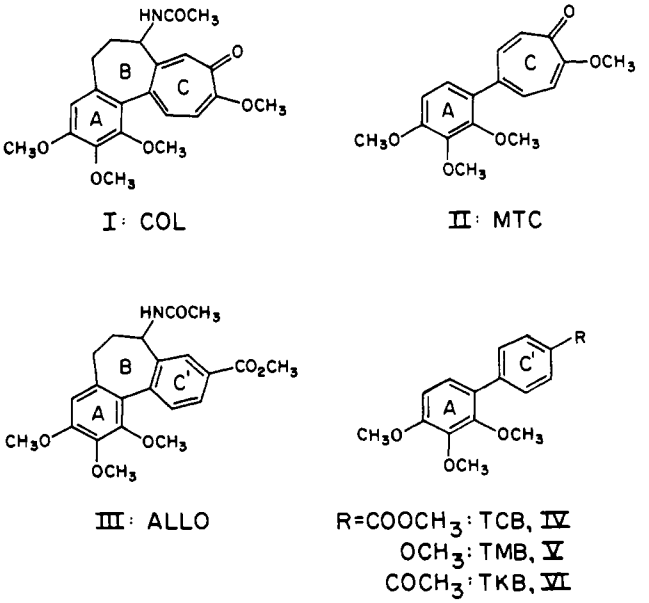
ABSTRACT: The roles of the oxygens in ring C of colchicine in its binding to tubulin were probed by a study of the interactions of two allocolchicine biphenyl analogues, 2,3,4,4′-tetramethoxy-1,1′-biphenyl (TMB) and 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl (TKB), the first one containing a methoxy group in position 4′, the second a keto group. Both analogues were found to bind specifically to the colchicine-binding site on tubulin in a rapidly reversible equilibrium. The standard free energies of binding at 25 °C were $\Delta G°$(TKB) = −7.19 ± 0.11 kcal mol^{-1} and $\Delta G°$(TMB) = −6.76 ± 0.22 kcal mol^{-1}. The binding of TKB induced the same perturbation in protein circular dichroism at 220 nm as colchicine and allocolchicine, as well as quenching of protein tryptophan fluorescence. Binding of TMB did not affect the protein CD spectrum within experimental error and induced only a marginal quenching of protein fluorescence. Comparison with the binding properties of allocolchicine and its des(ring B) analogue 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl (TCB) [Medrano et al. (1989) *Biochemistry 28*, 5589–5599] has shown that the binding properties of the 4′-keto analogue (TKB) were closer to those of allocolchicine, even though the substituent in the 4′-position of TCB is identical with that of allocolchicine. It has been proposed that binding in the ring C subsite on tubulin, which is stabilized thermodynamically by stacking interactions, can be modulated in a nonidentical fashion by the carbonyl and the ether oxygens in the para position of ring C.

The binding of colchicine to tubulin is a slow process that leads to the formation of a practically irreversible 1:1 complex (Garland, 1978). It has as consequences (i) perturbation of the tubulin far-UV circular dichroism spectrum (Andreu & Timasheff, 1982c); (ii) induction in tubulin of assembly-independent GTPase activity directed at the E-site-bound nucleotide (David-Pfeuty et al., 1979; Andreu & Timasheff, 1981); (iii) inhibition of microtubule assembly (Wilson & Bryan, 1974); and (iv) tubulin self-assembly into structures other than microtubules, but with the thermodynamic characteristics of microtubule growth (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982b; Andreu et al., 1983). The demonstration that binding to the colchicine site and its characteristic consequences are preserved both when ring B of colchicine (structure I, Chart I) is excised, with the formation of the compound 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (Fitzgerald, 1976)

Chart I



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‡ This paper and the following one are dedicated to Professor Robert H. Abeles on the occasion of his 65th birthday.
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(MTC)[1] (structure II) (Andreu et al., 1984; Bane et al., 1984), and when the tropolone methyl ether (ring C) of colchicine is rearranged to *p*-carbomethoxybenzene (ring C′) in allocolchicine (ALLO) (structure III) (Medrano et al., 1989) led to the examination of the simple biphenyl analogue of allocolchicine, 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl (TCB) (structure IV), with the finding that both binding specificity and its characteristic consequences were preserved (Medrano et al., 1989). The strength of the binding was reduced by 1.4 kcal/mol of standard free energy by the elimination of ring B and by 1.0 kcal/mol for the transformation of ring C into ring C′ (Medrano et al., 1989). The synthesis of the still simpler compound 2,3,4,4′-tetramethoxy-1,1′-biphenyl (TMB) (structure V) and the observation that it could also inhibit microtubule assembly (Medrano et al., 1986) prompted us to examine in depth the roles of the two oxygens of the substituent ($COOCH_3$) in the 4′-position of ring C′ of allocolchicine. To this end, a detailed examination of the binding properties to tubulin of TMB and 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl (TKB) (structure VI) was undertaken, and the consequences of their binding were scrutinized. The results of these studies, which define the roles of the carbonyl and methoxy oxygens, are described in this paper and the following one.

Materials and Methods

Ligands and Other Materials. Colchicine and podophyllotoxin were from Aldrich Chemical Co. 2-Methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC) was a gift from Dr. T. J. Fitzgerald (Fitzgerald, 1976). GTP dilithium salt was from Boehringer-Mannheim. Ethylenebis(oxyethylenenitrilo)tetraacetic acid and sodium dodecyl sulfate were from Sigma. Glycerol was from Merck, analytical grade, and all other chemicals were of reagent grade. The synthesis of TMB and TKB is described below. To check their purity, TKB and TMB were subjected to thin-layer chromatography (TLC) on 0.25-mm-thick silica gel plates (Fertigplatten, Merck) developed with chloroform–acetone–diethylamine (7:2:1) or dichloromethane–methanol (99:1). Both ligands gave a single spot under ultraviolet light. The ligands were dissolved in dimethyl sulfoxide and the resultant solutions stored at −20 °C. The residual dimethyl sulfoxide in the experiments was less than 1%, and these ligands could be dissolved to concentrations above 2×10^{-4} M in PG buffer. The concentrations of the ligands were measured spectrophotometrically. The extinction coefficients were determined by weighing dry crystals of the compounds with a Mettler UM-3 electrobalance, dissolving them gravimetrically in a final solution of PG buffer, and recording the UV spectrum. Four independent determinations gave $\epsilon_{295nm} = 14\,400 \pm 200$ M^{-1} cm^{-1} for TKB and $\epsilon_{256nm} = 16\,130 \pm 170$ M^{-1} cm^{-1} for TMB.

Synthesis of 2,3,4-Trimethoxy-4′-acetyl-1,1′-biphenyl (TKB). TKB was synthesized by a modified procedure of Elks and Hey (1943) for the synthesis of asymmetric biphenyls. Ten milliliters of freshly distilled trifluoroacetic acid was added with stirring over the course of 1 h to a mixture of 0.24 M 1,2,3-trimethoxybenzene with 0.055 M 1-(4-acetylphenyl)-3,3-dimethyltriazene kept at 70 °C. The mixture was then stirred for 1 h at 120 °C. After cooling, it was kept on water suction in vacuo at 45 °C for 1 h. The reaction residue was boiled with several portions of medium petroleum ether (MPE) (total 400 mL). The extract was washed with 5% $NaHCO_3$ and H_2O and dried over anhydrous $MgSO_4$ and K_2CO_3. Evaporation in vacuo at 45 °C gave 26.3 g of residue. The residue was sublimated at 500 μm at a temperature up to 100 °C. The nonsublimable material weighed 1.3 g. It was chromatographed on a column of neutral alumina (in MPE). Elution was carried out by slowly increasing the concentration of C_6H_6. The material eluted from the column gave an almost pure product (0.71 g, 4.5% yield). Recrystallization from MPE gave white needles. Mp: 103.0–103.5 °C (uncorrected). Anal. Found: C, 70.97; H, 6.31. Calcd: C, 71.31; H, 6.33. MS: MW 286 (theory 286), with a fragmentation pattern consistent with the structure of TKB. IR (CCl_4): 1678 (C=O); 1603 (aromatic); 1486 (aromatic); 1464 (aromatic); 1268 [C–(C=O)–C] cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.00 [d, J = 8.7 Hz, H-C(3′), H-C(5′)]; 7.62 [d, J = 8.7 Hz, H-C(2′), H-C(6′)]; 7.07 [d, J = 8.7 Hz, H-C(6)]; 6.77 [d, J = 8.7 Hz, H-C(5)]; 3.94 (s, ring A *m*-OCH_3); 3.92 (s, ring A *p*-OCH_3); 3.69 (s, ring A *o*-OCH_3); 2.64 (s, ketone CH_3). ^{13}C NMR ($CDCl_3$): δ 197.8 (C=O); 153.8 (C-2); 151.4 (C-4); 143.1 (C-1′); 142.6 (C-3); 135.3 (C-4′); 129.2 (C-2′, C-6′); 128.2 (C-3′, C-5′); 127.3 (C-1); 124.4 (C-6); 107.6 (C-5); 61.0 (2 ring A OCH_3); 56.0 (ring A OCH_3); 26.6 (ketone CH_3).

Synthesis of 2,3,4,4′-Tetramethoxy-1,1′-biphenyl (TMB). TMB was synthesized by the Ullman reaction using the previously described procedure (Medrano et al., 1989), with 0.02 M 1,2,3-trimethoxy-4-iodobenzene and 0.05 M 4-iodoanisole. The material eluted from the alumina column was rechromatographed on silica plates (PSC-Fertigplatten Kieselgel 60 F_{254}; E. Merck, Darmstadt) in CH_2Cl_2 to give TMB in a 29% yield. Recrystallization from MPE gave a crystalline material: Mp: 75.0–76.0 (uncorrected) [lit. mp 75 °C (Itoh et al., 1988)]. Anal. Found: C, 70.34; H, 6.68. Calcd: C, 69.96; H, 6.91. MS: MW 274 (theory 274), with a fragmentation pattern consistent with the structure of TMB. IR (CCl_4): 1600 (aromatic); 1490 (aromatic); 1245 (C–O); 1090 (C–O) cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.45 [d, J = 9.0 Hz, H-C(2′), H-C(6′)]; 7.02 [d, J = 8.4 Hz, H-C(6)]; 6.96 [d, J = 9.0 Hz, H-C(3′), H-C(5′)]; 6.74 [d, J = 8.7 Hz, H-C(5)]; 3.94 (s, ring A *m*-OCH_3); 3.91 (s, ring A *p*-OCH_3); 3.86 (s, ring C OCH_3); 3.68 (s, ring A *o*-OCH_3). ^{13}C NMR ($CDCL_3$): 158.5 (C-4′); 152.7 (C-2); 151.3 (C-4); 142.5 (C-3); 130.6 (C-1′); 130.1 (C-2′, C-6′); 128.3 (C-1); 124.5 (C-6); 113.6 (C-3′, C-5′); 107.4 (C-5); 61.0 (ring A OCH_3); 60.8 (ring A OCH_3); 56.0 (ring A OCH_3); 55.2 (ring C OCH_3).

Protein. Tubulin was purified from calf brains and stored in liquid nitrogen. It was prepared for use, and its concentration was measured as described previously (Weisenberg et al., 1968; Lee et al., 1973; Andreu et al., 1984). The tubulin–colchicine complex was prepared as described (Andreu & Timasheff, 1982c). All experiments were done in 10 mM sodium phosphate and 0.1 mM GTP, pH 7.0, buffer (PG buffer), at 25 °C, except where indicated.

Spectroscopic Measurements. Light absorption spectra, difference absorption spectra, and fluorescence and circular dichroism measurements were made as described previously (Medrano et al., 1989). Fluorescence emission intensities are given in the same arbitrary units as before (Medrano et al., 1989).

Binding Measurements. With these ligands it was not possible to do binding measurements by the equilibrium gel chromatography technique of Hummel and Dreyer (Hummel & Dreyer, 1962; Andreu, 1985; Medrano et al., 1989), as the

[1] Abbreviations: MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; ALLO, allocolchicine; TCB, 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl; TKB, 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl; TMB, 2,3,4,4′-tetramethoxy-1,1′-biphenyl; GTP, guanosine 5′-triphosphate; PG buffer, 10 mM sodium phosphate and 0.1 mM GTP, pH 7.0, buffer.

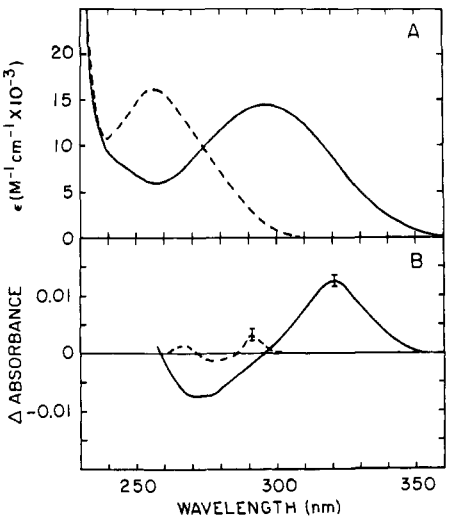

FIGURE 1: Panel A: absorption spectra of TKB (solid line) and TMB (dashed line). Panel B: difference absorption spectra generated by the interaction of tubulin with TKB and TMB in PG buffer, pH 7.0, at 25 °C. Solid line: difference spectrum of 8.95×10^{-6} M TKB and 1.03×10^{-5} M tubulin. Dashed line: difference spectrum of 3.53×10^{-5} M TMB and 1.11×10^{-5} M tubulin vs ligand and protein in separate solutions.

absorption spectra of these ligands have a large overlap with that of the protein and their fluorescence in organic solvents is very weak. For direct binding measurements we developed the following sedimentation method based on the procedure of Howlett et al. (1978). A solution of known concentrations of protein and ligand was centrifuged in 1-mL polycarbonate tubes (11 × 34 mm) in a TLA-100.2 rotor in the TL-100 Ultracentrifuge (Beckman) at 100000 rpm (356000*g*) for 2 h at the desired temperature. At the end of the centrifugation, the lower half of the tube contained the protein in equilibrium with free ligand; the upper half contained only free ligand and essentially no protein, as checked by control measurements. The upper 0.5 mL was withdrawn carefully and diluted to 1 mL with buffer, and the free ligand was measured spectrophotometrically. Because the ligands adsorb to the polycarbonate tubes, the total ligand concentration in solution was measured simultaneously in a tube centrifuged without protein, the upper and lower 0.5-mL portions were withdrawn carefully and diluted to 1 mL with buffer, and both were then measured spectrophotometrically. The ligand concentrations in the upper and lower fractions in the absence of protein were found to be the same within experimental error (3%). The amount of ligand adsorbed to the polycarbonate tubes varied between 6 and 25% of the total ligand concentration depending on the free ligand concentration. In each experiment the amount of ligand adsorbed to the tubes that corresponded to the free ligand concentration determined experimentally in the presence of protein was subtracted from the total concentration of added ligand. This gave the total ligand concentration available for the binding equilibrium to protein. The bound ligand concentration was taken as the difference between total concentration of ligand nonadsorbed to the tube and the free concentration. The values of the binding equilibrium constant and the number of sites were obtained from Scatchard plots of the data.

Quenching of the intrinsic protein fluorescence by ligand was also employed to estimate the binding affinity. The maximal fluorescence quenching by excess ligand was measured, and the fraction of sites occupied, α, was taken as equal to the fraction of the maximal quenching effect at a given total ligand concentration. The binding equilibrium constant was determined by employing the relationship $\alpha/(1-\alpha) = K_b[A]$, where [A] is the free ligand concentration taken as the difference of known total and bound ligand. The calculations were done assuming a 1:1 stoichiometry.[2] Binding measurements by fluorometric titration were made as described previously (Medrano et al., 1989). The fluorescence of free TKB was insignificant; that of bound TKB was weak, 1.2 ± 0.5 units/10^{-6} bound TKB[3] in PG buffer, pH 7.0, at 25 °C, with excitation at 315 nm and emission at 425 nm.

Binding was also measured indirectly by competition with a well-characterized reference ligand. The fractional saturation of the binding sites P by the reference ligand A (defined as $\alpha(A) = [PA]/[P]_0$) was measured as a function of the total concentration of competitor B. This was done at several total concentrations of A. The simultaneous equilibria are

$$P + A \rightleftarrows PA \quad (1)$$

$$P + B \rightleftarrows PB \quad (2)$$

giving rise to the following expressions:

$$K(A) = [PA]/[P][A] \quad (3)$$

$$K(B) = [PB]/[P][B] \quad (4)$$

$$[A] = [A]_0 - [PA] \quad (5)$$

$$[B] = [B]_0 - [PB] \quad (6)$$

$$[P] = [P]_0 - [PA] - [PB] \quad (7)$$

A personal computer program (J. F. Diaz, unpublished results) was employed to solve the system of equations (3–7) from the known values of $[A]_0$, $[B]_0$, $[P]_0$, and $K(A)$. The values of $K(B)$ were varied, in search of the value that renders a minimal sum of the squares of the deviations of the experimental from the theoretical values of $\alpha(A)$. In the present studies, the reference ligand employed was MTC, whose binding had been measured fluorometrically with $K(A) = 4.6 \times 10^5$ M^{-1} (Andreu et al., 1984). Simple graphical analyses of the competition, such as modified Dixon plots, were found to be inadequate because the free ligand concentrations were considerably smaller than the total concentrations added.

Miscellaneous Procedures. Colchicine binding to tubulin was followed by the fluorescence of bound colchicine (Bhattacharyya & Wolff, 1974; Andreu & Timasheff, 1982a,c). Sedimentation velocity experiments were performed in a Beckman Model E analytical ultracentrifuge. Identical samples, with and without ligand, were run simultaneously in double-sector cells in an An-D rotor at 60000 rpm.

Results

Determination of the Interaction of TKB and TMB with Tubulin by Difference Absorption and Fluorescence Spectroscopy. The interaction of tubulin with TKB and TMB was probed first by difference absorption spectroscopy. The direct absorption spectra in aqueous medium are shown in Figure 1A for TKB (solid line) and TMB (dashed line). They are characterized by maxima at 295 and 256 nm for TKB and TMB, respectively, and minima at 258 and 240 nm. The difference absorption spectra are displayed in Figure 1B, where the solid line is the difference spectrum generated by a solution

[2] A stoichiometry of 1:1 was assumed instead of the lower values obtained from other techniques (see Table I) so as to keep the various binding measurements independent of each other. The lower values of the stoichiometry, in fact, would not change significantly the resulting $\Delta G°$ of binding.

[3] This should be compared with 109 units/10^{-6} M bound allocolchicine (Medrano et al., 1989).

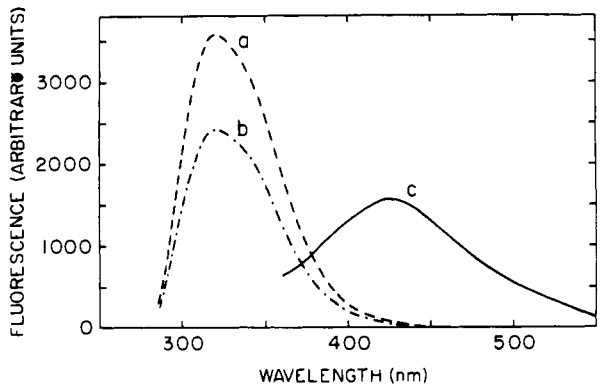

FIGURE 2: Fluorescence changes produced by the interaction of TKB with tubulin in PG buffer, pH 7.0, at 25 °C. Fluorescence emission spectra: (a) 7.3×10^{-6} M tubulin (excitation at 280 nm); (b) 7.3×10^{-6} M tubulin with 1.09×10^{-5} M TKB (excitation at 280 nm); (c) 3.88×10^{-5} M TKB with 6.3×10^{-6} M tubulin (excitation at 315 nm; spectrum amplified 125 times and base line corrected).

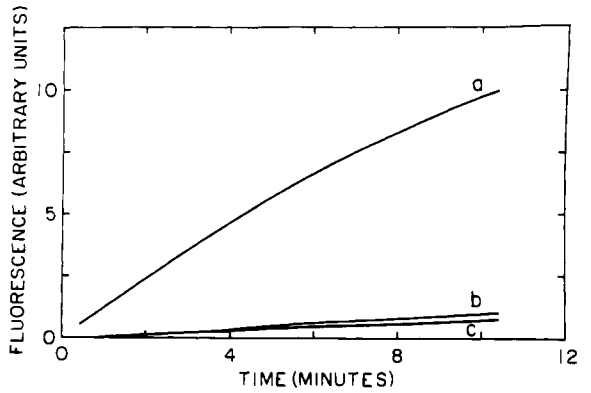

FIGURE 3: Inhibition of the initial binding rate of colchicine to tubulin by TKB and TMB at 25 °C. Fluorescence time courses of the binding of 1×10^{-5} M colchicine to 1.08×10^{-5} M tubulin in the absence (a) or in the presence of 1×10^{-4} M TMB (b) or TKB (c). The excitation wavelength was 365 nm, and the emission wavelength was 430 nm. The colchicine and TKB or TMB were added consecutively to the protein solution in the fluorometer cuvette as small volumes of concentrated solution. The ligands were mixed simultaneously to start the reaction.

of 8.95×10^{-6} M TKB and 1.03×10^{-5} M tubulin vs ligand and protein in separate solutions; the dashed line is the difference spectrum generated by 3.53×10^{-5} M TMB and 1.11×10^{-5} M tubulin. The spectrum generated by the interaction of tubulin and TKB is characterized by a maximum at 321 nm and a minimum at 274 nm. This difference spectrum is similar to the spectra of tubulin–allocolchicine and tubulin–TCB (Medrano et al., 1989). The interaction of tubulin and TMB generated only a weak difference spectrum characterized by maxima at 292 and 267 nm and a minimum at 279 nm.

The fluorescence of TKB is very weak in aqueous solution, and no fluorescence was detected with TMB. When TKB was bound to tubulin, it developed a weak fluorescence, as shown in Figure 2 (solid line), with an emission maximum at 425 nm. The large overlap of the excitation spectra of tubulin and TKB required that the ligand be excited at 315 nm, where there was no significant excitation of the protein. The ligand TMB did not exhibit any fluorescence signal in the presence of tubulin. The interaction of TKB with tubulin produced also a quenching of the intrinsic fluorescence of the protein, as shown clearly by comparison of the unliganded and liganded protein emission spectra (Figure 2, spectra a and b). This quenching effect is similar to that of the tubulin–colchicine complex (Andreu & Timasheff, 1982c) and to the quenching produced by MTC (Andreu et al., 1984). The ligand TMB also

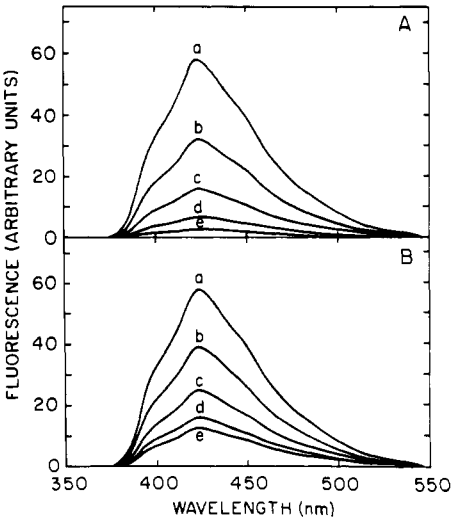

FIGURE 4: Inhibition of the equilibrium binding of MTC to tubulin by TKB and TMB. Panel A: base-line-corrected fluorescence emission spectra of 1.03×10^{-5} M MTC bound to 1.0×10^{-5} M tubulin in the absence (a) or in the presence of (b–e) 1.94×10^{-5}, 5.82×10^{-5}, 1.16×10^{-4}, or 1.94×10^{-4} M TKB. Panel B: fluorescence emission spectra of 1.03×10^{-5} M MTC bound to 1.0×10^{-5} M tubulin in the absence (a) or in the presence of (b–e) 2.0×10^{-5}, 6.0×10^{-5}, 1.2×10^{-4}, or 2.0×10^{-4} M TMB. The excitation wavelength was 350 nm. Identical results were obtained irrespective of the order of addition of the ligands. Equilibrium was attained in less than 2 min.

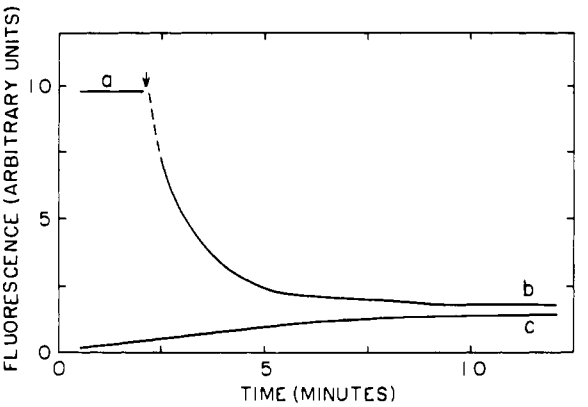

FIGURE 5: Fluorescence time course of TKB binding and dissociation. The reactions were started by addition of the ligand to the protein solution: (a) 1.94×10^{-5} M TKB and 6.6×10^{-6} M tubulin. After attainment of the plateau, 1×10^{-4} M podophyllotoxin was added to sample a producing tracing b. Tracing c is the same as tracing b, but podophyllotoxin was added 2 min before TKB.

quenched the intrinsic fluorescence of the protein (not shown), but the effect of this ligand was much weaker than that of TKB (ca. 7% of the protein fluorescence).

Specificity and Kinetics of the Interactions. The question whether the bindings of TKB and TMB were specifically directed to the colchicine-binding site in tubulin was addressed by competition experiments. The effects of the two ligands on the fluorescence time course of colchicine binding are shown in Figure 3. Tracing a is the fluorescence time course generated by the binding of 1×10^{-5} M colchicine to 1.08×10^{-5} M tubulin at 25 °C. Addition of 1×10^{-4} M TMB or TKB produced tracings b and c, respectively, which show that both ligands inhibit almost completely the binding of colchicine. The effects of the ligands on the binding of MTC, which binds specifically and in a rapidly reversible manner to the colchicine-binding site (Andreu et al., 1984), were also examined. The results, shown in Figure 4, clearly demonstrate that both TKB (panel A) and TMB (panel B) can compete with MTC for the binding to tubulin, as increasing amounts of the ligands

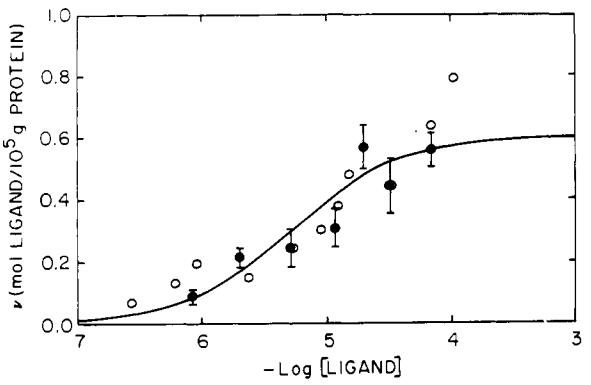

FIGURE 6: Binding isotherm of TKB to tubulin in PG buffer, pH 7.0, at 25 °C. The solid symbols are centrifugation measurements at protein concentrations of (4.7–5.6) × 10^{-6} M. The solid line is a fit to the centrifugation measurements (K_b = 1.91 × 10^5 M^{-1}; n = 0.60) obtained from a Scatchard plot of these data. The open symbols are ligand fluorescence binding measurements at 1.34 × 10^{-5} M protein, shown here simply for the purpose of comparison, since the low quantum yield of tubulin-bound TKB resulted in relatively large errors of measurement.

progressively reduced the fluorescence spectrum of the MTC bound to tubulin.

The binding time course of TKB to tubulin is shown in Figure 5, where tracing a was obtained on addition of 1.94 × 10^{-5} M TKB to 6.6 × 10^{-6} M tubulin. The emission intensity reached more than 95% of its maximal value in less than 30 s, similar to the binding of MTC (Andreu et al., 1984) and TCB (Medrano et al., 1989) to tubulin. The rate of reversibility of the tubulin–TKB complex was determined by displacement with podophyllotoxin. The result is shown in Figure 5, where at the point indicated by the arrow 1 × 10^{-4} M podophyllotoxin was added. The displacement of TKB (tracing b) was rapid, with a half-life of the order of 30 s. In the sample recorded as tracing c, tubulin was incubated for 2 min with 1 × 10^{-4} M podophyllotoxin prior to the addition of the ligand. The strong reduction of fluorescence shows blocking of the TKB-binding site by this competitor that shares the trimethoxybenzene ring with colchicine.

Binding Equilibrium Parameters. The quantitative characterization of the binding equilibrium of TKB to tubulin was performed by the centrifugation technique (see Materials and Methods). The binding isotherm of TKB to tubulin in PG buffer at pH 7.0 and 25 °C is shown in Figure 6, where the solid symbols are the individual centrifugation measurements and the solid line corresponds to a binding equilibrium constant, K_b = (1.91 ± 0.57) × 10^5 M^{-1}, and 0.60 ± 0.10 site/tubulin dimer, as estimated from a Scatchard plot of the data. The use of the centrifugation method raises two questions: What are the effects on the binding equilibrium parameters of protein aging during the experiment and of the large pressure applied? This was tested by applying the centrifugation method to the binding of MTC, an extensively characterized colchicine analogue (Andreu et al., 1984; Bane et al., 1984). The results obtained with this ligand at 25 °C were comparable to those reported previously; i.e., the binding parameters measured by centrifugation were K_b = 3.80 × 10^5 M^{-1} and n = 0.65 and by ligand fluorescence were K_b = 5.06 × 10^5 M^{-1} and n = 0.69, values that are essentially coincidental with the previously reported ones (Andreu et al., 1984), although the centrifugation method showed a trend toward slightly lower values of n and K_b. The results of approximate ligand fluorescence titrations (see Materials and Methods) performed under the same conditions are shown by the open symbols in Figure 6. Application of the centrifugation technique to the binding of TMB to tubulin gave high scatter due to the large errors caused by ligand adsorption to the polycarbonate tubes. Nevertheless, at TMB concentrations of ca. 10^{-4} M, it could be estimated that TMB binds to 0.7 ± 0.3 site on tubulin and to 0.00 ± 0.05 site on the tubulin–colchicine complex (data shown in the following paper). The results of binding measurements of TKB and TMB determined by the protein fluorescence quenching approach (see Materials and Methods) at various protein concentrations are shown in Figure 7. The equilibrium constant determined for TKB was K_b = (1.76 ± 0.25) × 10^5 M^{-1}. That for TMB, K_b = (1.31 ± 0.64) × 10^5 M^{-1}, was affected by a large error due to the overall small quenching effect, as reported above. The lack of any significant trend with protein concentration indicates that the binding of TKB or TMB to tubulin is not linked to a self-association reaction.

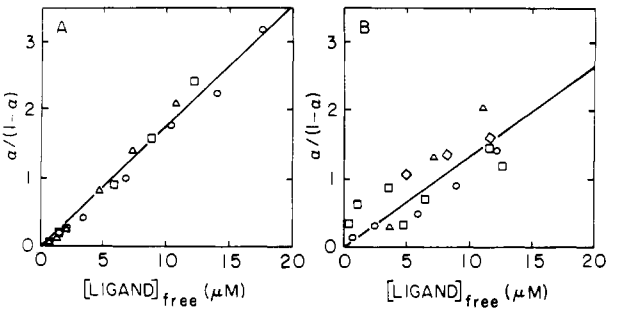

FIGURE 7: Protein fluorescence quenching titration of the binding of TKB (panel A) and TMB (panel B) to tubulin in PG buffer, pH 7.0, at 25 °C. Protein concentrations: panel A, (○) 0.14 mg mL^{-1}, (□) 0.39 mg mL^{-1}, and (△) 0.94 mg mL^{-1}; panel B, (□) 0.13 mg mL^{-1}, (△) 0.20 mg mL^{-1}, (○) 0.73 mg mL^{-1}, and (◇) 1.43 mg mL^{-1}. The straight lines are least-squares fits of the data.

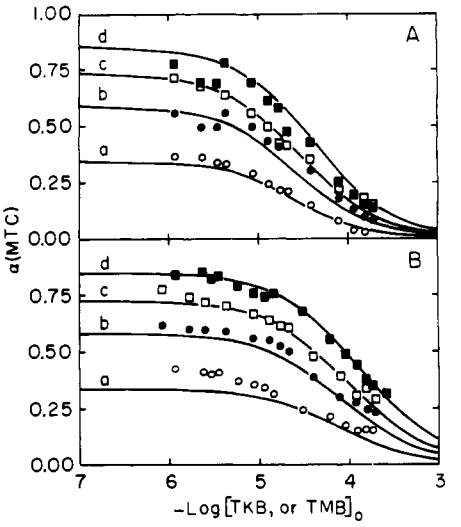

FIGURE 8: Competition of TKB (panel A) and TMB (panel B) with the reference ligand MTC for the binding to tubulin. The binding of MTC was measured fluorometrically as a function of total competitor concentration, and the results were analyzed as described under Materials and Methods. The total tubulin concentration was 1.3 mg mL^{-1}, and the total MTC concentrations were (a) 4.12 × 10^{-6} M; (b) 8.24 × 10^{-6} M; (c) 1.24 × 10^{-5} M; and (d) 2.00 × 10^{-5} M.

The binding of TKB and TMB to the colchicine site was also examined by competition with MTC, in experiments similar to those described in Figure 4. The inhibition of the equilibrium binding of MTC by TKB and TMB is displayed by Figure 8, where the lines show the best simultaneous fit to the complete set of data points (Materials and Methods). From these results, the estimated equilibrium association constants for TKB and TMB were (2.38 ± 0.65) × 10^5 and

Table I: Binding of TKB and TMB to Tubulin at 25 °C

	TKB				TMB			
method	protein concn (M)	$K_b \times 10^{-5}$ (M^{-1})	ΔG^0 (kcal/mol)	mol of ligand/10^5 g of tubulin	protein concn (M)	$K_b \times 10^{-5}$ (M^{-1})	ΔG^0 (kcal/mol)	mol of ligand/10^5 g of tubulin
centrifugation	4.7×10^{-6}–5.6×10^{-6}	1.91 ± 0.57	−7.21	0.60 ± 0.10	5.40×10^{-6}–5.60×10^{-6}	*b*		0.7 ± 0.3[d]
protein fluorescence quenching	1.4×10^{-6}–9.4×10^{-6}	1.76 ± 0.25	−7.16	*a*	1.30×10^{-6}–1.44×10^{-5}	1.31 ± 0.64	−6.98	*a*
ligand fluorescence	1.34×10^{-5}	1.42 ± 0.14	−7.03	0.75 ± 0.30		*c*		*c*
competition with MTC	1.1×10^{-6}–1.5×10^{-6}	2.38 ± 0.65	−7.34	*a*	1.21×10^{-6}–1.49×10^{-6}	0.63 ± 0.20	−6.55	*a*

[a] Information not afforded by this procedure; a 1:1 stoichiometry assumed. [b] Method not accurate for this ligand (see text). [c] Method not applicable to this ligand (see text). [d] At 10^{-4} M ligand.

Table II: Temperature Dependence of the Binding of TKB to Tubulin Measured by the Centrifugation Method

temp (°C)	$K_b \times 10^{-5}$ (M^{-1})	ΔG^0 (kcal/mol)	mol of ligand/10^5 g of tubulin
4	1.47 ± 0.61	−6.55	0.70 ± 0.10
10	1.72 ± 0.42	−6.78	0.64 ± 0.12
25	1.91 ± 0.57	−7.20	0.60 ± 0.10

(0.63 ± 0.20) × 10^5 M^{-1}, respectively. A summary of the results obtained by means of the different procedures is presented in Table I. Averaging the apparent standard free energy changes from the binding equilibrium constants obtained by means of the different procedures resulted in a value of −7.19 ± 0.11 kcal mol^{-1} for the interaction of TKB with tubulin and −6.76 ± 0.22 kcal mol^{-1} for the interaction of TMB with tubulin in PG buffer at pH 7.0 and 25 °C.

The binding of TKB to tubulin was found to be weakly dependent on temperature, as shown in Table II. The apparent standard free energy changes varied between −6.55 and −7.21 kcal mol^{-1} over the temperature range studied. This resulted in a $\Delta H°$ value not far from zero (1.9 ± 0.7 kcal mol^{-1}) and a $\Delta S°$ value of 30.5 eu.

Effects of Ligand Binding on the Conformation and Self-Association of Tubulin. The binding of colchicine and of previously studied analogues has been shown to result in a small perturbation of the protein circular dichroism in the far UV. Figure 9 shows the far-ultraviolet circular dichroism spectra of tubulin in the absence (tracing a) and in the presence (tracings b and c) of TKB and TMB and the calculated difference spectra (tracings d and e). In the case of TKB there was a small, but significant, ellipticity increment of −640 ± 200 deg cm^2 $dmol^{-1}$ near 220 nm. For TMB, the measured value was −70 ± 60 deg cm^2 $dmol^{-1}$ near 219 nm. $[\theta](220)/[\theta](210)$ changed from 1.035 ± 0.011 for unliganded tubulin to 1.114 ± 0.009 and 1.037 ± 0.012 in the presence of TKB and TMB, respectively. The small change induced by TKB is similar to those previously reported for tubulin liganded to colchicine (Andreu & Timasheff, 1982c), MTC (Andreu et al., 1984), allocolchicine (Medrano et al., 1989), and tropolone methyl ether (Andreu & Timasheff, 1982c). The very small effect measured with TMB is within experimental error and should be regarded as insignificant.

The effects of TKB and TMB binding on tubulin self-association were also examined. TKB or TMB (1.5×10^{-4} M) did not induce any significant changes in the sedimentation velocity of 5.16 mg/mL tubulin in PG buffer, pH 7.0, at 20 °C. This indicates the absence of ligand-induced tubulin self-association under the conditions employed to measure binding.

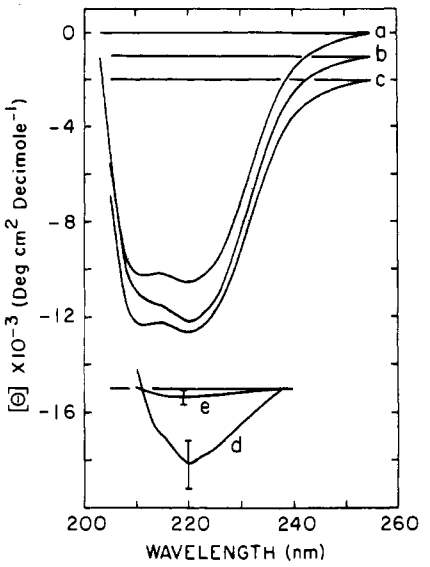

FIGURE 9: Effect of ligands on the circular dichroism spectrum of tubulin in PG buffer, pH 7.0, at 20 °C: (a) spectrum of (1.55–1.69) × 10^{-5} M tubulin; (b) spectrum of 1.55 × 10^{-5} M tubulin and 1.94 × 10^{-5} M TKB; (c) spectrum of 1.69 × 10^{-5} M tubulin and 2.00 × 10^{-5} M TMB. Each spectrum is the average of duplicate samples. Tracings d and e are the result of subtracting tracing a from tracings b and c, respectively, amplified 5 times.

Discussion

The above results firmly establish that the simple biphenyl colchicine analogues TKB and TMB bind to tubulin specifically into the colchicine binding site, as both inhibited colchicine binding and competed with MTC. Since the trimethoxyphenyl ring (ring A of colchicine) is common to the two compounds and, therefore, interacts with the trimethoxyphenyl-specific subsite on tubulin, the differences in properties observed must all stem from the interaction of ring C′ with the tropolone methyl ether binding subsite on tubulin. Comparison of the binding characteristics points to significant differences in the patterns of local interactions. The parent analogue for the two compounds is TCB, which contains both the carbonyl and the methoxy oxygens in ring C′. Looking at the spectroscopic data, of the two derivatives only the one that contains the carbonyl oxygen, TKB, has the same general characteristics as TCB. In fact, it resembles allocolchicine in its general behavior better than TCB does. Its binding was accompanied by the generation of a difference spectrum similar to that obtained on the binding of TCB or allocolchicine to tubulin and of a difference circular dichroism spectrum of the same character and magnitude as those generated by the binding of allocolchicine to tubulin. The methoxy analogue,

on the other hand, generated only a very weak difference spectrum on binding to tubulin and a weak quenching of the protein fluorescence and perturbed the protein far-UV ellipticity only marginally, if at all. These differences in the spectroscopic consequences of the binding of TKB and TMB to tubulin may reflect a displacement of the TMB absorption band to higher energy, as well as nonidentity of the orientations of the transition dipoles of ring C′ in the two analogues with respect to those of the pertinent aromatic residue in tubulin, since the effects of ring A should have remained invariant.

The standard enthalpy change of the binding of ring C to tubulin is negative (Andreu & Timasheff, 1982a). While this is consistent with hydrogen-bond formation between the oxygens of the ligand and pertinent donor groups in the protein binding site, the magnitude of $\Delta H°$ is too great. The spectroscopic data, on the other hand, can be explained in terms of the stacking of ring C with aromatic protein residues (Andreu & Timasheff, 1982c; Rava et al., 1987; Hastie, 1989; Hastie & Rava, 1989), and the thermodynamic parameters of the binding of ring C to tubulin (Andreu & Timasheff, 1982c) are most consistent with π-stacking interactions (Engelborghs, 1981). A further contribution that might be considered is that of electrostatic interactions between aromatic groups when arranged in an edge-to-face fashion, i.e., with their planes perpendicular to each other (Burley & Petsko, 1988). This, however, being a quadrupole–quadrupole interaction, can be expected to be low.

Following the examination of a large number of synthetic analogues directed at the colchicine-binding site on tubulin, Itoh et al. (1989) have concluded that a methoxy substituent in position 4′ of ring C′ in biphenyls is required for these compounds to bind to tubulin and to have a significant effect on tubulin polymerization. A more rigorous examination, however, reveals that the situation is more complex, since the analogue with a methyl ketone in position 4′ mimics colchicine better. In fact, the present results suggest that there are two modes of interaction in subsite C, governed by the nature of the hydrogen-bond acceptor of the substituent in the 4′-position of ring C′. The first mode, determined by the presence of a carbonyl group in position 4′, results in a perturbation of the protein CD spectrum at 217–220 nm. This can reflect a change in the secondary structure either of the protein or of the asymmetric environment of a tyrosine or tryptophan residue that has chiral bands in that spectral region (Brahms & Brahms, 1980). The strong quenching of protein fluorescence, which accompanies the binding of the carbonyl analogue, suggests energy transfer and is consistent with a stacking interaction with a tryptophan residue in the protein. The second mode, defined by the presence of an ether (—O—) oxygen in position 4′, neither induces the change in ellipticity, suggestive of the perturbation of the secondary or tertiary structures of the protein, nor leads to strong quenching of protein fluorescence. Both would be consistent with a normal, rather than parallel, mutual alignment of the transition dipoles of the ring C′ phenyl and the aromatic group in the protein. Further light can be shed on this dichotomy in the modes of binding by comparison with the binding properties of TCB. This analogue contains both the carbonyl and methoxy oxygens. It could be expected, therefore, to bind to tubulin in a mode closest to that of allocolchicine. Yet, this is not the case, as TKB mimics allocolchicine best: The binding affinities of the analogues are $\Delta G_b° = -8.1$ kcal mol^{-1} for allocolchicine, -7.2 kcal mol^{-1} for TKB, and -6.8 kcal mol^{-1} for TCB and TMB. The protein circular dichroism perturbation induced by TCB is weak, less than half of those induced by TKB and allocolchicine, which are essentially identical, and the wavelength maximum of the TCB difference CD spectrum is shifted slightly from those of TKB and allocolchicine, the difference CD spectra for which are essentially identical. These similarities of the binding properties of allocolchicine and TKB would suggest that it is the formation of a hydrogen bond to the carbonyl group that locks ring C in the tubulin subsite in the mode in which it enters into a stacking interaction with an aromatic chromophore on the protein, while hydrogen bonding to the ether oxygen (TMB) does not give the same locking. The results with TCB are unexpected. It can form hydrogen bonds in both the —C=O and —O— modes. The observations suggest that it either occupies the subsite partially in the two modes or can fluctuate between them. In order to test further the modulation of the mode of ring C binding by the substituent in the 4′ biphenyl position, a number of additional analogues were synthesized and the results are the subject of the following paper.

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Registry No. TMB, 6271-59-6; TKB, 132491-63-5; colchicine, 64-86-8; 1,2,3-trimethoxybenzene, 634-36-6; 1-(4-acetylphenyl)-3,3-dimethyltriazene, 52416-18-9.

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Mechanism of Colchicine Binding to Tubulin. Tolerance of Substituents in Ring C′ of Biphenyl Analogues[†,‡]

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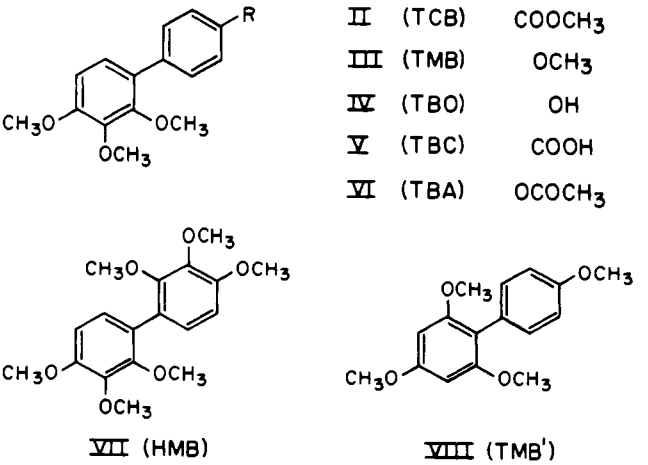
ABSTRACT: The limits of structural variation of the substituent in position 4′ of ring C′ of biphenyl colchicine analogues (ring C in colchicine) were probed by the synthesis of a number of analogues and the examination of their binding to tubulin and its consequences. Binding was found to require the location in three-dimensional space of the oxygen in the 4′-substituent at a locus not far distant from those of the colchicine ring C oxygens. All those analogues that bind to the colchicine site of tubulin induced the GTPase activity and inhibited microtubule assembly, those containing a carbonyl group substoichiometrically and the others stoichiometrically. A similar relation was found for the induction of the abnormal polymerization of the colchicine analogue–tubulin complex, with methoxy-containing compounds requiring a higher temperature to induce the polymerization. A concerted analysis of the binding thermodynamics of colchicine and its various analogues has shown full consistency with the previously proposed two-step binding pathway that involves two nonidentical binding moieties in the ligand [Andreu, J. M., & Timasheff, S. N. (1982) *Biochemistry 21*, 534–543]. Comparison of the binding parameters of colchicine, its des(ring B) analogue (MTC), and ring A and C compounds individually with the thermodynamic parameters deduced for the first steps of the bindings of colchicine and MTC [Engelborghs, Y., & Fitzgerald, T. J. (1987) *J. Biol. Chem. 262*, 5204–5209] have led to the conclusion that binding can occur by two pathways leading to the identical product. In the first pathway, ring A binds first; this is followed by a rate-determining thermodynamically indifferent reaction (protein conformation change), and finally a rapid binding of ring C. In the second pathway, the events are the same except that the order of binding of the rings is reversed. Colchicine, due to the steric hindrance of ring B, can follow only the second pathway. For MTC, both kinetic pathways are open and binding may be initiated by random first contact of either ring A or ring C.

The finding that the binding pattern of biphenyl colchicine analogues was controlled by the nature of the oxygen-containing group in position 4′ of ring C′, carbonyl or ether, led us to investigate the limits of variation in this substituent that would still be compatible with binding into the colchicine site on tubulin. The similarity to the binding pattern of allocolchicine diminished in this order: carbonyl (TKB)[1] (structure I, Chart I) > carbomethoxy (TCB, structure II) > methoxy (TMB, structure III), in position 4′ (Medrano et al., 1991). As a result, the substituent in this position was varied further, and the effects on binding to tubulin were examined. The new compounds included 2,3,4-trimethoxy-1,1′-biphenyl-4′-ol (TBO, structure IV), the phenol derivative of TMB, 2,3,4-trimethoxy-1-1′-biphenyl-4′-carboxylic acid (TBC, structure V), the free acid derivative of TCB, and

Chart I



VII (HMB) VIII (TMB′)

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[‡] This paper and the preceding one are dedicated to Professor Robert H. Abeles on the occasion of his 65th birthday.

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