Structural Analysis of the Substoichiometric and Stoichiometric Microtubule-Inhibiting Biphenyl Analogues of Colchicine†

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ABSTRACT: The structures of the colchicine (COL) analogues, 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl (TKB) and 2,3,4,4′-tetramethoxy-1,1′-biphenyl (TMB), were solved by X-ray diffraction. Their comparison with the structure of colchicine indicated the ability of both compounds to enter into a colchicine binding pocket. Comparison of TKB with 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl (TCB) showed that the methyl group of the carbomethoxy group in position 4′ of TCB protrudes beyond the (C═O)−CH_3 group in the same position in TKB. Superposition of both structures on the van der Waals surface of COL clearly demonstrates that TKB can fully fit within that domain, while the CH_3 group of TCB protrudes beyond the COL contour. This is proposed to be the source of the inability of TCB to inhibit microtubule assembly substoichiometrically, while TKB is a very strong inhibitor. While the same steric hindrance to entering into the COL site on tubulin must exist in allocolchicine (ALLO), in its case, this is overcome by the rigidity of the three-ring structure which abolishes the loss on binding of the entropy of free rotation between the two rings of the biphenyl TCB.

In the preceding paper (Perez-Ramirez et al., 1996), a detailed analysis of microtubule assembly inhibition by colchicine and its analogues has led to the conclusion that substoichiometric inhibition requires the presence of a carbonyl group in the proper position on ring C of colchicine (or ring C′ of the allocolchicine family of compounds) (structures shown in that paper). Allocolchicine (ALLO),[1] which is a substoichiometric inhibitor, contains a carbomethoxy group ($COOCH_3$) in position 4′ of ring C′.[2] Its biphenyl analogues, TKB and TMB, which, in turn, contain the keto or the methoxy group in that position, were found to be a strong and a weak substoichiometric inhibitor, respectively. Furthermore, the analogue (TCB) that contains the same carbomethoxy group as ALLO was found to be a stoichiometric inhibitor. Since TCB contains a carbonyl group in the same position (4′) as TKB, it was proposed that substoichiometric inhibition requires the formation of a contact (perhaps a hydrogen bond) between the carbonyl oxygen and the proper site on the protein that could be penetrated by the methyl ketone of TKB, the penetration of which by TCB was inhibited sterically by the greater length of the carbomethoxy group. In order to verify this hypothesis, the structures of the biphenyl analogues TKB and TMB were determined by X-ray crystallography. These structures, as well as that of TCB (Mackay et al., 1989b), were compared and projected onto that of colchicine to define the extra bulkiness that causes the proposed steric inhibition. The present paper describes the results.

EXPERIMENTAL PROCEDURES

The biphenyl analogues TKB and TMB were synthesized as described previously (Medrano et al., 1991). Crystals for both compounds were obtained after a few days from a refrigerated solution in petroleum ether. Final cell parameters and other information regarding the data collection procedure are given in Table 1.

Data for TMB were collected using a Rigaku AFC5R diffractometer with a rotating anode copper radiation source. Data for TKB were collected on a Syntex diffractometer (Crystal Logic) with a regular copper X-ray source. The data for both compounds were corrected for Lorentz and polarization effects. Azimuthal scans of several reflections were performed and resulted in transmission factors ranging from 0.94 to 1.00. An empirical absorption correction was performed on data obtained from TMB crystals. Both data sets were put on an absolute scale with a Wilson plot.

TMB was solved by direct methods using SHELXS (Sheldrick, 1985), and almost all the heavy atoms were found initially. Isotropic and then anisotropic full-matrix least squares refinements converged, and a difference Fourier synthesis was calculated. The resulting electron density map

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[1] Abbreviations: COL, colchicine; ALLO, allocolchicine; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; 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; TME, tropolone methyl ether.

[2] The position numbering in ALLO was chosen for consistency with the biphenyl compounds. It differs from the standard numbering in the colchicine series of compounds.

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Table 1: Experimental Details

	TMB	TKB
(A) Crystal Data		
formula	$C_{16}H_{18}O_4$	$C_{17}H_{18}O_4$
formula weight	274.32	286.33
crystal color, habit	colorless, block	colorless, plate
crystal dimensions (mm)	0.40 × 0.35 × 0.30	0.30 × 0.30 × 0.40
crystal system	monoclinic	monoclinic
number of reflections used for unit cell determination (2θ range, deg)	25 (46.2−63.7)	15 (28.0−46.3)
lattice parameters		
a	14.715(2) Å	18.494(3) Å
b	7.292(2) Å	14.328(3) Å
c	14.944(2) Å	12.404(2) Å
β	118.64(6)°	116.44(4)°
V	1407.2(4) Å^3	2943.1 Å^3
space group	$P2_1/n$ (#14)	$C2/c$ (#15)
Z	4	8
F(000), e	584	1216
μ (cm^{-1})	7.7	0.99
d_{calcd} (g cm^{-3})	1.29	1.29
(B) Intensity Measurements		
diffractometer	Rigaku AFC5	Syntex (Crystal Logic)
radiation	Cu Kα (=1.541 78 Å)	Cu Kα
temperature (°C)	23	23
scan type	$\omega - 2\theta$	$\theta - 2\theta$
scan rate	32.0°/min (in ω) (two rescans)	12°/min
$2\theta_{max}$	120.2°	115°
number of reflections measured	2387 (total)	2252 (total)
	2289 (unique)	2183 (unique)
	(R_{int} = 0.026)	(R_{int} = 0.045)
number of reflections refined	1735	1795
number of refined parameters	190	192
observations per parameter	9.1	9.3
R	0.058	0.066
R_W	0.084	0.090

revealed the positions of most of the ring hydrogen atoms and some of the methyl hydrogen atoms, which were added to the list of atomic parameters. The rest of the methyl group hydrogens were included at calculated positions with a C−H distance 0.96 Å. Hydrogen atoms were not refined. At about $R = 9\%$, a difference Fourier map was examined and showed the existence of disorder about one of the methoxy methyl groups, C(7). Two positions for this atom, C(7) and C(17), were included in subsequent calculations, each at an occupancy of 0.5. The hydrogen atoms for this disordered methyl group were not calculated. The quantity minimized in the least squares refinement for TMB was $\sum\omega(|F_o| - |F_c|)^2$, where the weights $\omega = a + F_o + cF_o^2$, $a = 2F_o$(min), and $c = 2/F_o$(max) and with zero weight for those reflections below the threshold of $I > 3\sigma I$ (Cruickshank, 1965). $\boldsymbol{R} = \sum||F_o| - |F_c||/\sum F_o$, where F_o and F_c are, respectively, an observed structure factor and one calculated from a postulated structure. Hence, *R* is a measure of the extent to which the measured diffraction pattern of a structure agrees with that calculated from the parameters of the structure determined by analysis of the diffraction data. Computer programs used were from the CAOS crystallographic library (Cerrini & Spagna, 1977).

For TKB, the direct methods program SHELXS provided the correct structure solution, and all the heavy atoms were found initially. Most of the hydrogen atoms were found from a difference Fourier, the rest were calculated, and none of the hydrogen atoms was refined. The weights minimized in this structure refinement were $\omega = 1/\sigma^2$ and with zero weight for those reflections below the threshold $I > 3\sigma I$. Computer programs used were from the UCLA Program Library (Byrn & Strouse, 1991). Tables of structure factors and anisotropic temperature factors for both TMB and TKB are available from M. Rossi.

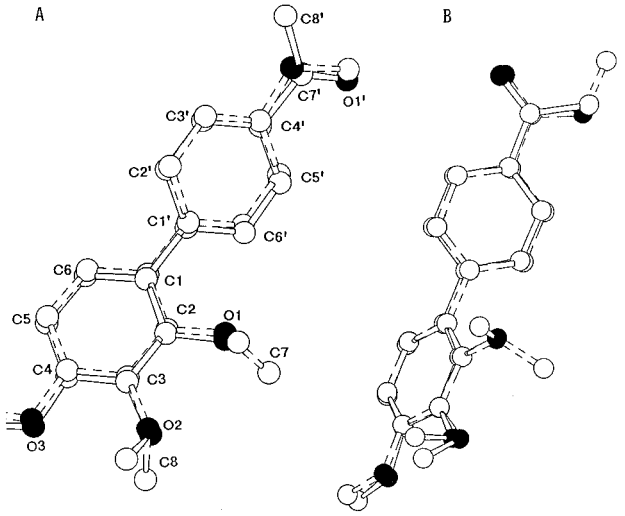

FIGURE 1: (A) Structures of TKB (solid) and TMB (dashed) superimposed on each other. (B) TKB rotated (solid) and superimposed on the structure of TCB (dashed) (note the coincidence of the carbonyl oxygens).

RESULTS AND DISCUSSION

Structures of TKB and TMB. The structures of TKB and TMB are shown in Figure 1. Tables of the atomic coordinates and of crystallographic F_o (*hkl*) and F_c (*hkl*) of TMB and TKB are available from M. Rossi and from Cambridge Structural Data Base. Distances and angles for TMB and TKB lie within expected values and show no

unusual features. For both compounds, the C(1)−C(1′) bond distances are statistically the same: 1.486(5) Å in TMB and 1.473(4) Å in TKB. In TKB, the distance C(4′)−C(7′) is 1.476(4) Å, while the C(7′)−C(8′) distance is 1.523(8) Å. Within experimental error, the C(4′)−C(7′) distance is shorter than C(7′)−C(8′) and contributes significantly to the resonance between the carbonyl C(7′)−O(1′) group and the attached C(1′)···C(6′) phenyl group, making them almost coplanar (167.2°). The two phenyl rings in TMB and TKB do not deviate from planarity, and this may be seen from calculations of distances from each of the atoms in the respective phenyl rings to the best calculated plane through those six carbon atoms.

The overall conformation of the two molecules is such that the twist angle between the trimethoxy ring (ring A) and the second ring (ring C′) in TMB is 48.6(4)° and in TKB is 44.0(2)°. Although these angles are smaller than those for similar compounds, this shows the same disposition of the rings A and C (or C′) as seen in three-ring colchicine analogues: colchicine, 51 and 53° (Lessinger & Margulis, 1978a); isocolchicine, 53 and 57° (Lessinger & Margulis, 1978b); and allocolchicine, 48.7(4)° (Mackay et al., 1989a). Other two-ring colchicine analogues [rings A and C (or C′) only] also show a similar disposition between the A and C (or C′) rings. In 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC), the angle between the two planes is 57.4° (Rossi et al., 1984). For the 2,3,4-trimethoxy-3′(and also 4′)-carbomethoxy-1,1′-biphenyl compounds (Mackay et al., 1989b), the angles are 55.3(3)° for the 3′-carboxylic acid derivative and 59.1(5) and 63.8(5)° for the two molecules in the 4′-carboxylic acid isomer (TCB). As noted earlier, it appears as though ring B has little effect on the orientation of rings A and C (or C′) in these compounds.

The structures of TMB and TKB, shown superimposed on each other in Figure 1A, display remarkably similar overall conformations. Figure 2A,B shows these molecules superimposed on the structures of one of the two molecules in the asymmetric unit of colchicine (the two independent molecules in the asymmetric unit of colchicine are similar in conformation). This comparison reveals that the dispositions of the different functional groups in ring C′ of TKB ($COCH_3$) and TMB (OCH_3) are such that the oxygen atoms of TKB and TMB are in close proximity to the two functional groups of ring C of colchicine: the carbonyl oxygen and methoxy oxygen. In these superpositions, the displacements of the analogue oxygens from the space loci of the two colchicine ring C oxygens are 0.9 Å from the COL carbonyl and 1.0 Å from the COL methoxy for TMB and 0.7 Å from the COL carbonyl and 0.7 Å from the COL methoxy for TKB. Proper rotation of TKB into a low-energy conformation that is only 0.5 kcal higher in energy than the X-ray structure conformation reduces to 0.1 Å the displacement of the TKB carbonyl oxygen from the COL carbonyl oxygen. A similar superposition was performed for TCB on COL, shown in Figure 2C. The displacement of the TCB oxygens from their counterparts in COL are 0.1 Å for the carbonyl and 0.2 Å for the methoxy.

Comparison of these structures and their superpositions on COL shows that both TKB and TMB have an oxygen that is not far displaced from the space locus of the COL carbonyl. Yet their microtubule-inhibiting capacities are drastically different. A possible explanation for this might be found in the hydrogen-bonding capacities of a keto and an ether oxygen (Joesten & Schaad, 1974), which would lead to a stronger anchoring of TKB.

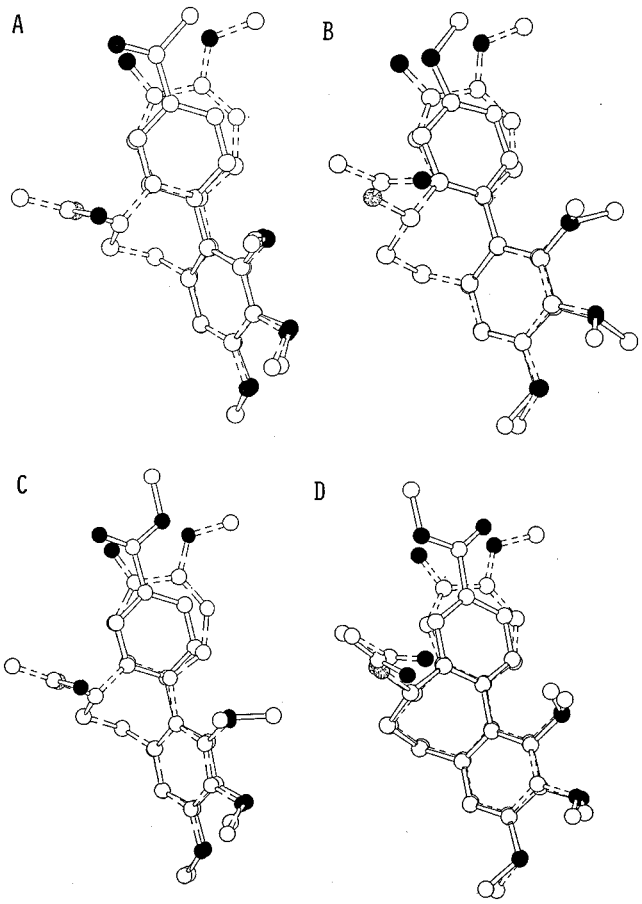

FIGURE 2: Superpositions of the structures of analogues on that of colchicine. (A) TKB rotated as described in the text. (B) TMB. (C) TCB. (D) ALLO. In all cases, the structure of COL is dashed, and that of the analogue is solid.

A similar comparison was carried out between TKB and TCB. Their superposition, given in Figure 1B, shows clearly that the carbonyl groups of both molecules occupy the same locus in space and the two are identically related to the position of the COL carbonyl. Furthermore, TCB has a methoxy oxygen positioned close to the COL methoxy (Figure 2C). Why then is TCB a weak, stoichiometric inhibitor while TKB is a strong, substoichiometric one? The answer can be found by examination of Figures 1B and 2A,C. These figures show that, while both rings A and C′ of TKB and TCB fit exactly into a COL binding pocket on the protein, the same is not true of the group attached to position 4′ on ring C′. Superposition of TCB on TKB in Figure 1B reveals that TCB has a methyl group that protrudes beyond the methyl group of TKB. The steric consequences are strikingly brought into evidence if the TCB and TKB structures are superimposed on the van der Waals surface of COL, as shown in Figure 3A,B. It is clear that the TCB methyl group extends beyond the structural domain of COL, while TKB can be completely accommodated into this domain. If substoichiometric inhibition requires fitting of ring C into a tight pocket that can just accommodate COL, the protrusion on ring C′ of TCB may generate sufficient steric hindrance to prevent that molecule from penetrating fully into this part of the binding pocket. No such hindrance should occur with TKB as is evident from Figure 3A. A

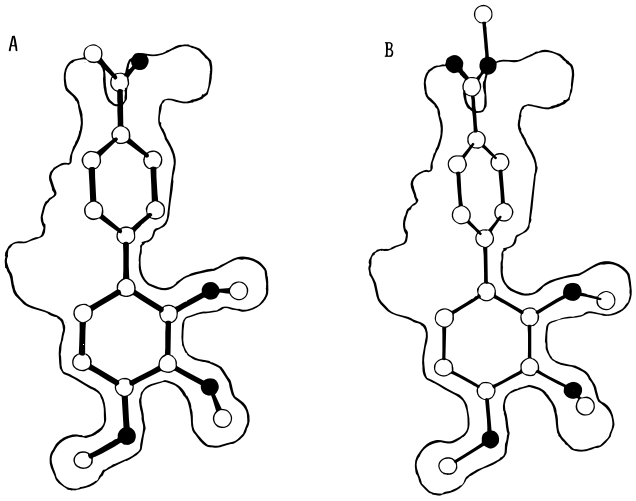

FIGURE 3: Fitting of analogues within the colchicine van der Waals surface. (A) TKB. (B) TCB.

cursory examination of Figures 2C and 3B may suggest that rotation about the C(7)-methoxy oxygen bond should bring the ester methoxy of TCB into a configuration that could be accommodated into the region within the van der Waals surface of COL that is filled by the comparable methyl of COL. This cannot happen, however, because, in such a configuration, there would be severe strain due to the steric hindrance between the hydrogens of the ester methyl and the phenyl hydrogen of ring C′.[3] It should be noted furthermore that structures having a $COOCH_3$ substituent on a phenyl ring always assume the same conformation as shown for TCB in Figures 2C and 3B.

In light of these findings, the following question must be answered. Why is allocolchicine (ALLO) a substoichiometric inhibitor of microtubule assembly? It carries the same group in position 4′ as TCB, and as shown in Figure 2D, it displays the same protrusion of the position 4′ methoxy beyond the van der Waals surface of COL. Otherwise, it fits well into a COL domain. The answer must be found in the rigidity of the three-ring ALLO structure; TCB, on the other hand, has only two rings (A and C′), which permits free rotation between them. If induction of substoichiometric inhibition requires the COL family of molecules to penetrate within a pocket and be locked in specific steric orientation, TKB has the steric ability to do this. The anchoring that takes place through the van der Waals, and probably stacking, interactions of ring C′ with the proper groups in the tubulin pocket (Andreu & Timasheff, 1982b; Hastie, 1989; Hastie & Rava, 1989), as well as between its carbonyl and a hydrogen bond donor in the pocket (Andreu & Timasheff, 1982a,b), is evidently sufficiently strong to overcome the loss of the entropy of rotation between rings A and C′. TCB, however, is unable to form sufficiently strong interactions both to compensate for the loss of rotational entropy and to overcome the steric strain related to penetration into the pocket. The binding of ALLO, on the other hand, does not involve the loss of rotational entropy, since ring C′ is held rigidly in proper orientation by ring B. This results in a gain of ca. -2 kcal mol^{-1} of free energy (Glasstone, 1940; Orville-Thomas, 1974; Benson, 1976; Andreu et al., 1991). Coupled with the ensuing formation of a hydrogen bond inside the pocket, this may be sufficient to compensate for the unfavorable contribution from the steric strain.

[3] A computer-generated drawing of the TCB structure (Andreu et al., 1991) was shown previously with the CH_3 group rotated toward the phenyl ring. This structure is incorrect since that group cannot occupy the drawn conformation because of the steric interference discussed in the text.

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