Alternative Binding Modes for Chloramphenicol and 1-Substituted Chloramphenicol Analogues Revealed by Site-Directed Mutagenesis and X-ray Crystallography of Chloramphenicol Acetyltransferase[†,‡]

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ABSTRACT: Leucine-160 of chloramphenicol acetyltransferase (CAT) has been replaced by site-directed mutagenesis to investigate enzyme–ligand interactions at the 1-hydroxyl substituent of the substrate chloramphenicol. The consequences of the substitution of Leu-160 by glutamine and by phenylalanine were deduced from the steady-state kinetic parameters for acetyl transfer from acetyl-CoA to the 3-hydroxyl of chloramphenicol and its analogues 1-deoxychloramphenicol and 1-acetylchloramphenicol. The acetyl group of the latter, which is a substrate both in vivo and in vitro, could potentially bind in a similar position to the 1-hydroxyl of chloramphenicol, in close proximity to the side chain of Leu-160. In the case of Gln-160 CAT, large increases in K_m for the three acetyl acceptors were accompanied by small decreases in k_{cat} and in apparent affinity for acetyl-CoA. Such results are consistent with the introduction of the relatively hydrophilic amide in place of the δ-methyl groups of Leu-160. The kinetic properties of Phe-160 CAT were unexpected in that K_m for each of the three acetyl acceptors was unchanged or reduced, compared to the equivalent parameters for the wild-type enzyme, whereas k_{cat} fell significantly (44–83-fold) in each case. The ratios of specificity constants (k_{cat}/K_m) for the acetylation of chloramphenicol compared with the alternative acyl acceptors were similar for wild-type and mutant enzymes. As the residue substitutions for Leu-160 do not result in enhanced discrimination against the binding and acetylation of 1-acetylchloramphenicol, it appears unlikely that the 1-acetyl group binds to the CAT active site in the same position as that occupied by the 1-hydroxyl of chloramphenicol. The structure of the binary complex of chloramphenicol and Phe-160 CAT was determined at 2.0-Å resolution and found to be essentially isosteric with that of wild-type CAT. However, the position of the bound chloramphenicol differs in Phe-160 CAT due to a 2.0-Å movement of the 1-hydroxyl into a novel, more hydrophilic, environment. This results in new van der Waals contacts between the aryl group of the substrate and the side chains of Phe-160 and Ile-172. As a consequence, the critical distance between the 3-hydroxyl of chloramphenicol and $N^{\epsilon 2}$ of the imidazole of His-195 is extended from 2.8 to 4.7 Å so that the observed mode of binding is almost certainly nonproductive. An additional binding mode for chloramphenicol, which is likely to be similar to but not identical with that which occurs in wild-type CAT, must be invoked to account for the observed activity of the Phe-160 mutant.

Bacterial resistance to the antibiotic chloramphenicol (Figure 1), an inhibitor of the peptidyl transferase activity of prokaryotic ribosomes, is commonly conferred by the enzyme chloramphenicol acetyltransferase (CAT,[1] EC 2.3.1.28; Shaw, 1967), which catalyzes acetyl transfer from acetyl-CoA to the 3-hydroxyl of chloramphenicol. The initial product of the reaction, 3-acetylchloramphenicol, can undergo nonenzymic intramolecular rearrangement to 1-acetylchloramphenicol, which is a substrate for a second cycle of enzymic acetylation at the 3-hydroxyl to yield 1,3-diacetylchloramphenicol as the final product (Scheme I). Both of the mono- and diacetylated derivatives of chloramphenicol fail to bind bacterial ribosomes and are therefore devoid of antibiotic activity (Shaw & Unowsky, 1968).

Kinetic characterization of the catalytically efficient enterobacterial type III variant (CAT_{III}) shows that the reaction proceeds via a random order, rapid equilibrium, ternary complex mechanism. $N^{\epsilon 2}$ of the imidazole group of His-195[2] is thought to act as a general base in the abstraction of a proton from the 3-hydroxyl of chloramphenicol, thereby promoting

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[‡] Crystallographic coordinates of the Leu-160 → Phe mutant of chloramphenicol acetyltransferase have been submitted to the Brookhaven Protein Data Bank under the name 4CLA.

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; CAT_{III}, type III variant of CAT, *cat*, gene encoding CAT; CM, chloramphenicol; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MPD, 2-methyl-2,4-pentanediol; TLC, thin-layer chromatography; TMS, trimethylsilane; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TSE buffer, 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 0.1 mM EDTA.

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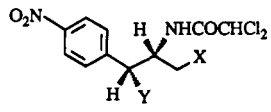


X	Y	COMPOUND
-OH	-OH	Chloramphenicol
-OH	-H	1-Deoxychloramphenicol
-OH	-OAc	1-Acetylchloramphenicol
-I	-OH	3-Iodochloramphenicol
-I	-OAc	1-Acetyl-3-iodochloramphenicol
-I	$-OCOCH_2CH_3$	1-Propionyl-3-iodochloramphenicol

FIGURE 1: Structure of chloramphenicol and selected analogues.

nucleophilic attack at the acetyl-CoA thioester (Kleanthous & Shaw, 1984; Klenthous et al., 1985). The structures of the binary complexes of CAT_{III} with chloramphenicol and with CoA have been solved at 1.75- and 2.4-Å resolution, respectively (Leslie et al., 1988; Leslie, 1990). The enzyme is a trimer with active sites lying at each of the three interfaces between adjacent subunits. The majority of the residues forming the chloramphenicol binding site are contributed by one subunit whereas the imidazole of His-195 is provided by the adjacent monomer (Figure 2).

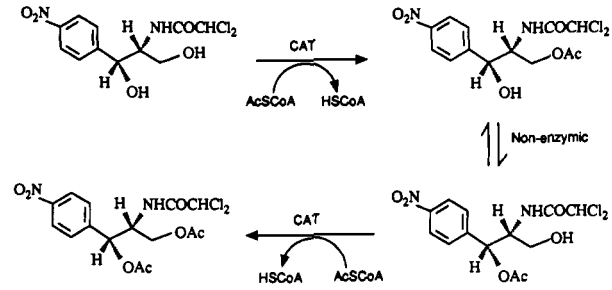
The gene encoding CAT_{III} has been cloned and overexpressed in high yield in *Escherichia coli* (Murray et al., 1988), facilitating studies by site-directed mutagenesis. The latter have addressed questions relating to enzyme stability (Lewendon et al., 1988), transition-state stabilization (Lewendon et al., 1990), the role of electrostatic and hydrophobic interactions in coenzyme A binding (Day, 1990), and the properties of a cysteinyl residue in the chloramphenicol binding site of CAT_{III} (Lewendon & Shaw, 1990).

The secondary acetylation cycle catalyzed by CAT, which is not required for the resistance phenotype, might be expected to confer a disadvantage to a bacterial cell since two molecules of acetyl-CoA are consumed where one would suffice. Nonetheless, the ability to acetylate 1-acetylchloramphenicol is common to CAT variants that are highly divergent at the level of primary structure. In the present study we have attempted to analyze the factors determining the specificity of CAT_{III} for the C1-substituent of chloramphenicol by using the complementary techniques of steady-state kinetic analysis, substitution of the 1-hydroxyl of the acetyl acceptor, site-directed substitutions of Leu-160, which forms part of the chloramphenicol binding site of CAT, and X-ray crystallography of a binary complex between chloramphenicol and the L160F mutant of CAT.

In the binary complex of CAT_{III} and chloramphenicol, the substrate lies in a deep pocket at the subunit interfaces. There are extensive van der Waals contacts between chloramphenicol and side chains lining the pocket, but the 1-hydroxyl group lies in a small cavity situated at the base of the pocket and formed by atoms in the side chains of Ser-148 (C^{β}), Leu-160 ($C^{\delta 1}$), Ile-172 ($C^{\gamma 2}$), and Thr-174 ($O^{\gamma 1}$) (Figure 3). This cavity is occupied by three water molecules, Wat249, Wat252, and Wat360, one of which (Wat252) forms a bridging hydrogen bond between the 1-hydroxyl and the hydroxyl group of Thr-174, one of only three hydrogen bonds between enzyme and substrate (Leslie, 1990). Ser-148, Leu-160, and Thr-174 are conserved residues, whereas Ile-172 is replaced by Val in some variants of CAT (Shaw et al., 1979; Murray et al., 1990). Modeling 1-acetylchloramphenicol in place of chloramphenicol

[2] Alignment of the amino acid sequences of 10 CAT variants has resulted in a general numbering system, which is used here. Tyr-25, Asn-146, Ser-148, Phe-158, Leu-160, Ile-172, Thr-174, and His-195 are residues 20, 140, 142, 152, 154, 166, 168, and 189, respectively, in the primary sequence of CAT_{III} (Murray et al., 1988).

Scheme I: Enzymic and Nonenzymic Relationships between Chloramphenicol and Its Acetylated Adducts



indicated that the acetyl substituent could be accommodated within the cavity by displacing the bound water and by a minor rearrangement of substrate or enzyme to obviate unfavorable van der Waals contacts (data not shown). However, it has not been possible to obtain crystals of a binary complex between CAT and either 1-acetylchloramphenicol or 1,3-diacetylchloramphenicol as a consequence of the acyl migration reaction and because the 3-acetyl substituent is subject to slow enzymatic hydrolysis in the absence of CoA. We therefore employed site-directed mutagenesis to test the hypothesis that 1-acetylchloramphenicol binds to the enzyme with the acetyl moiety positioned within the cavity. Of four possible candidate residues, Leu-160 was chosen as the most suitable for substitution, thus avoiding disruption of the hydrogen bond to Thr-174 and the loss of the contribution of the side chain of Ser-148 to transition-state stabilization (Lewendon et al., 1990). Leu-160 was replaced by glutamine or phenylalanine, the former being approximately isosteric with leucine but hydrophilic and likely to affect the organization of water molecules within the proposed 1-acetyl site, whereas the L160F substitution was expected to impose a largely steric barrier to binding of the acetylated substrate.

In the first instance, the steady-state kinetic parameters were determined with wild-type CAT for the 3-O-acetylation of chloramphenicol and analogues wherein the 1-hydroxyl was replaced by a proton (1-deoxychloramphenicol) or substituted by an acetyl group (1-acetylchloramphenicol). Such studies provide quantitative data for the second acetylation reaction, which had previously been described only by the analysis of chloramphenicol acetylation products by TLC (Shaw, 1967; Thibault et al., 1980). The analysis was extended to the mutant enzymes to establish whether the two residue substitutions for Leu-160 resulted, as predicted, in enhanced discrimination against 1-acetylchloramphenicol as substrate when compared with chloramphenicol or 1-deoxychloramphenicol. Competitive inhibitors wherein the 3-hydroxyl of chloramphenicol is replaced by iodine and the 1-hydroxyl is substituted by acetyl or propionyl groups were also employed to determine the tolerance of wild-type CAT for increasing chain length of the C1 subsituent.

Previous studies (Lewendon et al., 1988; Gibbs et al., 1990) revealed extensive and unexpected structural changes as a consequence of single point mutations of CAT_{III} that could not easily have been predicted by computation or modeling. The structure of Phe-160 CAT was determined at 2.0-Å resolution to ensure that the structural consequences of the residue substitution were limited to the immediate environment of the chloramphenicol binding site and to assist interpretation of the kinetic data derived for the mutant enzymes.

Taken together, the results of the present study show that the active site of CAT_{III} is remarkably tolerant in its capacity to accommodate (and acetylate) chloramphenicol and its 1-substituted analogues. At least two novel binding modes for

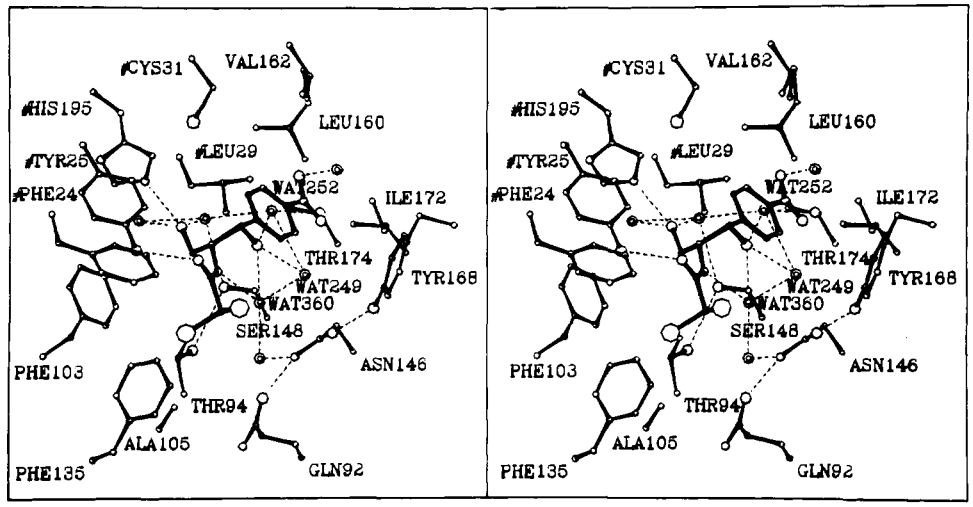


FIGURE 2: Stereoview of residues forming the chloramphenicol binding pocket of wild-type CAT_{III}. Carbon, nitrogen, oxygen, sulfur, and chlorine atoms are drawn as circles of increasing size. Ordered water molecules are shown as double circles and possible hydrogen bonds are indicated by broken lines. Residue names preceded by # belong to an adjacent subunit of the trimer.

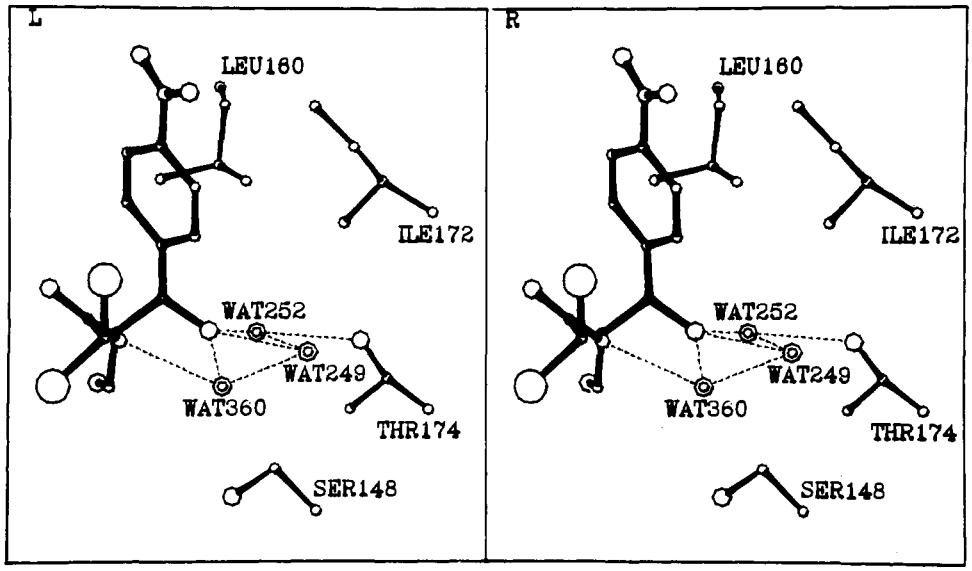


FIGURE 3: Stereoview of residues in close proximity to the 1-hydroxyl group of chloramphenicol. Ordered water molecules are shown as double circles and possible hydrogen bonds are indicated by broken lines.

the acetyl acceptor are suggested by the results of the kinetic and structural analyses, in addition to that which pertains in the binary complex of chloramphenicol and wild-type CAT.

Experimental Procedures

Site-Directed Mutagenesis and Expression of CAT. Oligonucleotide-directed mismatch mutagenesis was performed according to the deoxyuridine selection protocol with the *dut ung E. coli* strain RZ1032 (Künkel et al., 1987). The presence of the desired nucleotide substitutions and the absence of second-site mutations were confirmed by determination of the nucleotide sequence of the DNA spanning the entire *cat* coding sequence and 5′ noncoding regions. Mutant and wild-type *cat* determinants were overexpressed in *E. coli* following transfer to plasmid pUC18 (Murray et al., 1988).

Purification of CAT. Purification of wild-type and mutant CAT from *E. coli* extracts was carried out by using affinity chromatography on chloramphenicol–Sepharose as previously described (Lewendon et al., 1988). The purity of enzyme preparations was assessed by SDS–polyacrylamide gel electrophoresis wherein each CAT protein produced single bands of identical mobility.

Assay of CAT Activity. CAT activity was assayed spectrophotometrically at 25 °C. One unit of enzyme activity is defined as the amount converting 1 μmol of chloramphenicol to product per minute.

(*i*) *Forward Reaction.* The procedure described by Lewendon et al. (1990) was used to measure rates of acetylation of chloramphenicol. The standard assay contained TSE buffer, pH 7.5, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.1 mM chloramphenicol, and 0.4 mM acetyl-CoA. The reaction was initiated by addition of enzyme, and the formation of CoA was monitored at 412 nm. Concentrations of acetyl-CoA and the different acetyl acceptors (chloramphenicol, 1-deoxychloramphenicol, or 1-acetylchloramphenicol) were varied in the standard assay during kinetic analyses. In the case of 1-acetylchloramphenicol, the reaction was initiated by addition of acetyl acceptor as a 50-fold concentration in dry acetonitrile to limit isomerization prior to mixing. Control experiments showed that CAT activity was unaffected by the presence of 2% acetonitrile in the assay.

(*ii*) *Kinetic Analysis.* Linear initial rates for steady-state kinetic analyses were measured in triplicate over times during which less than 15% depletion of substrates occurred. Kinetic parameters were determined from linear slope and intercept

replots from manually drawn double-reciprocal plots (Kleanthous & Shaw, 1984). Concentrations of inhibitors and chloramphenicol were varied in the standard assay during competitive inhibition studies that routinely included acetyl-CoA at a fixed concentration $\simeq 5$ times K_m. The K_i values were determined from linear slope replots derived from double-reciprocal plots as described.

NMR Spectroscopy. NMR spectra were recorded on a Varian EM390 90-MHz spectrometer, with TMS as internal standard.

Synthesis of Substrate Analogues

(*i*) *1-Deoxychloramphenicol.* L-Phenylalaninol (5 g, 33 mmol) was dissolved in dry pyridine (70 mL) and cooled to 0 °C, and acetic anhydride (12.5 mL, 132 mmol) was added. After standing at 0 °C for 16 h, the solution was poured into 200 mL of water and extracted with 3 × 250 mL of ethyl acetate, and the organic phase was washed with dilute HCl, aqueous $NaHCO_3$, and then water. The organic phase was dried with $MgSO_4$ and evaporated to yield *N,O*-diacetylphenylalaninol as a white crystalline solid. *N,O*-Diacetylphenylalaninol (3 g, 12.7 mmol) was added in portions to stirred fuming nitric acid (10 mL, −7 °C) over 30 min. After a further 10 min the solution was allowed to warm to room temperature, decanted onto ice (30 g), neutralized with excess solid $NaHCO_3$, and extracted with 3 × 250 mL of ethyl acetate. After being washed with water and dried with $MgSO_4$, the organic phase was evaporated to give the crystalline product *p*-nitro-*N,O*-diacetylphenylalaninol. The acetyl substituents were hydrolyzed by refluxing in 5% HCl (80 mL) for 2 h, followed by cooling and extraction into ether (2 × 250 mL). The pH was raised to 11 by the addition of 1 M NaOH, and the product was extracted into ethyl acetate, followed by evaporation to yield the free amine. This was refluxed in dry ethanol (80 mL) containing 5.6 g of methyl dichloroacetate for 45 min, followed by rotary evaporation to remove volatile components. The faintly yellow crystalline residue was triturated with petroleum ether, then dissolved in ethyl acetate, washed with dilute HCl, aqueous $NaHCO_3$, and water, and then dried with $MgSO_4$ and evaporated to give a crystalline solid. Recrystallization from aqueous ethanol gave 1-deoxychloramphenicol as fine white needles with a yield of 1.1 g. 1-Deoxychloramphenicol: mp 147.5–148.5 °C; ^{1}H NMR (DMSO-d_6/$CDCl_3$) δ 2.9–3.2 (m, 2 H), 3.6 (m, 2 H), 3.9–4.3 (m, 1 H), 4.7 (br t, 1 H, D_2O exch), 6.0 (s, 1 H), 7.3 (d, 2 H), 7.8–8.2 (m, 3 H, one of which slow D_2O exch).

(*ii*) *1-Acetylchloramphenicol.* Chloramphenicol was converted to the *threo*-2-dichloromethyl-4-(*p*-nitro-α-hydroxybenzyl)-Δ^2-oxazoline according to Farkas and Sicher (1953). The oxazoline was dissolved in pyridine and treated with acetic anhydride (2 equiv) at 0 °C for 16 h. The solution was diluted with water and extracted with ethyl acetate. The organic phase was washed with dilute HCl and then aqueous $NaHCO_3$, dried with $MgSO_4$, and then evaporated to give the oxazoline acetate. This was converted to 1-acetylchloramphenicol by refluxing for 30 s in 96% ethanol containing 5% concentrated HCl. After cooling, neutralization with bicarbonate, and the addition of ethyl acetate, the organic phase was evaporated to yield 1-acetylchloramphenicol. 1-Acetylchloramphenicol was stored in solution at 4 °C in dry acetonitrile to avoid isomerization to 3-acetylchloramphenicol. 1-Acetylchloramphenicol: colorless oil; ^{1}H NMR ($CDCl_3$) δ 2.1 (s, 3 H), 2.5 (br t, 1 H, D_2O exch), 3.4–3.8 (m, 2 H), 4.1–4.5 (m, 1 H), 5.9 (s, 1 H), 6.2 (d, 1 H), 7.0 (br d, 1 H, slow D_2O exch), 7.6 (d, 2 H), 8.2 (d, 2 H).

Synthesis of Competitive Inhibitors

(*i*) *3-Iodochloramphenicol.* Chloramphenicol (10 g, 31 mmol) was dissolved in 50 mL of dry pyridine and cooled to 0 °C. *p*-Toluenesulfonyl chloride (6.7 g, 35 mmol) was added with stirring over 20 min and stirred for 2 h. The solution was poured into 150 mL of cold water, and the resulting oil was extracted with 3 × 150 mL of ethyl acetate and washed with dilute HCl, aqueous $NaHCO_3$, and brine. The organic phase was dried with $MgSO_4$ and evaporated to yield 3-(*p*-toluenesulfonyl)chloramphenicol, which was dissolved in 150 mL of acetone and refluxed, with constant stirring, for 6 h with sodium iodide (15 g) under a nitrogen atmosphere. After cooling to 0 °C and filtration to remove sodium *p*-toluenesulfonate, the solvent was evaporated and the residue was dissolved in 200 mL of ethyl acetate. The solution was washed with 100 mL of 10% aqueous sodium thiosulfate and then water (2 × 100 mL), dried with $MgSO_4$, and then evaporated to yield 3-iodochloramphenicol, which was subsequently recrystallized from 67% aqueous ethanol. 3-Iodochloramphenicol: mp 138–139 °C; ^{1}H NMR ($CDCl_3$) δ 3.1–3.6 (m, 2 H), 4.2–4.5 (m, 1 H), 5.3 (br s, 2 H, one of which slow D_2O exch), 6.0 (s, 1 H), 7.3–7.6 (m, 3 H, one of which slow D_2O exch), 8.2 (d, 2 H).

(*ii*) *1-Acetyl-3-iodochloramphenicol.* 3-Iodochloramphenicol (2 g, 4.6 mmol) was dissolved in 30 mL of dry pyridine, cooled to 0 °C, and treated with acetic anhydride (0.95 mL, 10 mmol). After 24 h the solution was added to 200 mL of water and extracted with 2 × 60 mL of ethyl acetate. The organic phase was then washed with dilute HCl and aqueous $NaHCO_3$ (2 × 50 mL of each) and dried with $MgSO_4$ before evaporation to yield the desired product, which was recrystallized from 67% aqueous methanol. 1-Acetyl-3-iodochloramphenicol: mp 75–78 °C; ^{1}H NMR (DMSO-d_6/$CDCl_3$) δ 2.16 (s, 3 H), 2.9–3.5 (m, 2 H), 4.2–4.5 (m, 1 H), 5.9 (s, 1 H), 6.1 (d, 1 H), 7.0 (br d, 1 H, slow D_2O exch), 7.6 (d, 2 H), 8.25 (d, 2 H).

(*iii*) *1-Propionyl-3-iodochloramphenicol.* The compound was prepared in an identical manner with 1-acetyl-3-iodochloramphenicol but with propionic rather than acetic anhydride. 1-Propionyl-3-iodochloramphenicol: mp 74–78 °C; ^{1}H NMR ($CDCl_3$) δ 1.1 (t, 3 H), 2.4 (q, 2 H), 3.2–3.8 (m, 2 H), 4.4–4.8 (m, 1 H), 5.9 (s, 1 H), 6.1 (d, 1 H), 7.0 (br d, 1 H, slow D_2O exch), 7.6 (d, 2 H), 8.2 (d, 2 H).

Crystallization and Structure Determination of Phe-160 CAT. Single crystals were grown by microdialysis in small Lucite "buttons". Each button contained 30 μL of protein (5 mg mL^{-1}) in 10 mM MES, pH 6.3, and was dialyzed against 3 mL of 5% MPD, 10 mM MES, pH 6.3, 1 mM chloramphenicol, 0.5 mM hexammine cobalt(III) chloride at 4 °C (Leslie et al., 1986). Crystals were harvested into the same solution as the dialysate but containing 8% MPD. The crystals are isomorphous with those of the wild-type enzyme, space group *R*32, with hexagonal cell parameters a = 107.7 Å and c = 123.9 Å (wild-type values are 107.6 and 123.4 Å, respectively).

X-ray data to 2-Å resolution were collected by using the image plate scanner at the EMBL outstation of the DESY synchrotron in Hamburg, with radiation of wavelength 1.0 Å. The data were collected in two runs. The first used a rotation angle of 1.6°/image and an exposure of 4000 counts/step. The second run was designed to measure reflections that were overloaded on the first. The detector to crystal distance was adjusted to give a maximum resolution of 2.6 Å, and a rotation of 2.5°/image and exposure of 250 counts/step were used. All data were collected from one crystal. The images were pro-

Table I: Refinement Statistics for Phe-160 CAT

Diffraction Data	
reflections (6–2.0 Å)	17970 (99.1% complete)
R-value[4]	14.7%

atomic model	no. of atoms	mean isotropic thermal parameter (Å^2)
protein	1711	20.4
chloramphenicol	20	13.6
solvent	205	48.5

stereochemical refinement parameter	rms deviation from ideal values	refinement restraint weighting values
bond distances (Å)	0.021	0.02
angle distances (Å)	0.041	0.03
planar 1–4 distances (Å)	0.062	0.05
planes (Å)	0.020	0.03
chiral volumes (Å^3)	0.22	0.15
van der Waals single torsion (Å)	0.16	0.2
multiple torsion (Å)	0.16	0.2

cessed with a modified version of the film processing package MOSFLM. The modifications (due to H. Terry and S. McLaughlin) allow for the fact that whereas the optical densities are stored as bytes for film data, the larger dynamic range of the image plate scanner necessitates the use of 16-bit words. The crystallographic merging R-factor[3] was 6.5% for the 2-Å resolution images (9.3% at 2 Å), 4.2% for the 2.6-Å resolution images (9.3% at 2.6 Å) and 3.8% for merging the 2-Å and 2.6-Å resolution data. The refined 1.75-Å resolution wild-type structure (Leslie, 1990) was used as an initial model for phase calculation and gave an initial R-factor[4] of 21.8% for all data between 6- and 2-Å resolution. An electron density map with coefficients $(3F_o - 2F_c)\alpha_c$ showed a significant shift in the position of bound chloramphenicol. The position of the substrate was adjusted manually to fit the electron density, and three water molecules in steric conflict with this new position were deleted from the model prior to refinement. The structure was refined by using the restrained parameter least-squares refinement program PROLSQ (Hendrickson & Konnert, 1980), and following refinement the model was adjusted manually by using the interactive graphics program FRODO (Jones, 1978). Two rounds of alternate refinement and manual rebuilding resulted in a final model with good stereochemistry (Table I) and a crystallographic R-factor of 14.7%[4] for all data between 6- and 2-Å resolution. The appearance of the chloramphenicol density and the values of the refined temperature factors (Table I) both suggest that chloramphenicol is present at full occupancy.

Results and Discussion

Kinetics of Acetylation of Alternative Acyl Acceptors by Wild-Type CAT. Steady-state kinetic parameters for the acetylation of 1-deoxyCM and 1-acetylCM by wild-type CAT_{III} are summarized in Table II. Values of k_{cat}/k_m for the acetylation of chloramphenicol and 1-deoxychloramphenicol are similar at 5.2×10^7 M^{-1} s^{-1} and 1.4×10^7 M^{-1} s^{-1}, respectively. However, the specificity constant for 1-acetylchloramphenicol (3.7×10^5 M^{-1} s^{-1}) is ~150-fold lower than that for chloramphenicol, confirming the relative inefficiency of the second acetylation cycle to give the diacetyl product. Discrimination against 1-acetylchloramphenicol results primarily from a decrease (~40-fold) in k_{cat} rather than an increase in the K_m of the acyl acceptor. A possible explanation is that the side chain of Ser-148 is displaced in order to avoid contact with the 1-acetyl moiety of the substrate, thereby disrupting coordination of the oxyanion of the transition state (Lewendon et al., 1990). Alternatively, the position of the substrate in the binary complex may differ from that normally occupied by chloramphenicol such that the critical interactions with Ser-148 or His-195 are destabilized.

Binding of Competitive Inhibitors to Wild-Type CAT. Although the proposed "1-hydroxyl cavity" of CAT might accommodate an acetyl group with relative ease, modeling studies suggested that the binding of the larger propionyl moiety would require a major displacement of the remainder of chloramphenicol. To test the tolerance of the enzyme for increasing chain length of the acyl C1 substituent, a series of potential competitive inhibitors were prepared wherein the 3-hydroxyl of chloramphenicol was replaced by iodine and the C1 substituent was either a hydroxyl, acetyl, or propionyl group. As the binding of chloramphenicol and its 3-substituted analogues by CAT conforms to a linear free energy relationship determined by the relative hydrophobicity of the C3 substituent (Cullis et al., 1991) it is probable that 3-iodochloramphenicol and chloramphenicol bind to the enzyme in a similar manner. The K_i values for 3-iodochloramphenicol, 1-acetyl-3-iodochloramphenicol, and 1-propionyl-3-iodochloramphenicol are 3.7 μM, 7.2 μM, and 29 μM, respectively. The observed high affinity for the latter compound suggests that the chloramphenicol binding site of CAT_{III} is surprisingly tolerant in its response to substitution of the 1-hydroxyl of the substrate and supports the proposition that 1-acyl derivatives of chloramphenicol may bind in a different configuration from that observed in the CAT/chloramphenicol binary complex (Leslie, 1990).

Kinetic Characterization of Gln-160 CAT. The kinetic properties of Gln-160 CAT for the acetylation of chloramphenicol, 1-deoxychloramphenicol, and 1-acetylchloramphenicol are summarized in Table III. Values of k_{cat}/K_m for the acetylation of chloramphenicol, 1-deoxychloramphenicol, and 1-acetylchloramphenicol are 1.5×10^6 M^{-1} s^{-1}, 4.1×10^5 M^{-1} s^{-1}, and 9.2×10^3 M^{-1} s^{-1}, respectively—a decrease of 34–40-fold compared with the equivalent parameters for wild-type CAT in each case (see above). Since the binding of chloramphenicol by CAT has been shown to be dominated by hydrophobic interactions (Cullis et al., 1991), the increased K_m values for chloramphenicol and its analogues are consistent with the enhanced hydrophilicity of the binding site caused by the amide substitution. Similarly, as the side chain of Leu-160 (via $C^{\delta 2}$) is thought to contribute to the binding of the acetyl groups of the tetrahedral intermediate and of acetyl-CoA (P. C. E. Moody and A. G. W. Leslie, unpublished results), the observed minor decreases in k_{cat} and affinity for the acyl donor are readily explicable.

The ratio of specificity constants for the conversion of a substrate and an analogue, which is itself a substrate, provides a convenient estimate of the extent of discrimination achieved by an enzyme active site. For Gln-160 CAT these values are 3.7 and 163 for 1-deoxychloramphenicol and 1-acetylchloramphenicol, respectively, compared with 3.7 and 149 for wild-type CAT, indicating that the residue substitution has neither enhanced nor impaired discrimination against the alternative substrates.

Kinetic Characterization of Phe-160 CAT. The kinetic properties of Phe-160 CAT (Table III) were unexpected, as

[3] The merging R factor (R_{merge}) is defined as $R_{merge} = \sum\sum|I(h)_i - I(h)|/\sum\sum I(h)_i$, where $I(h)_i$ is the scaled intensity of the reflection h from the ith film, $I(h)$ is the weighted mean of all observations of reflection h, and the summation includes all observations.

[4] Progress of the structure refinement was monitored by a reliability index, R, defined as $R = \sum|F_o - F_c|/\sum F_o$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively.

Table II: Kinetic Parameters Determined for Acetyl Transfer to Chloramphenicol, 1-Deoxychloramphenicol, and 1-Acetylchloramphenicol by Wild-Type CAT[a] and for Competitive Inhibition by Chloramphenicol Analogues[b]

acetyl acceptor (*)	k_{cat} (s^{-1})	K_m* (μM)	K_m (acetyl-CoA (μM)	k_{cat}/K_m* (M^{-1} s^{-1})	relative specificity[c]	inhibitor	K_i (μM)
chloramphenicol	599	11.6	93.4	5.2×10^7		3-iodochloramphenicol	3.7
1-deoxychloramphenicol	311	23	151	1.4×10^7	3.7	1-acetyl-3-iodochloramphenicol	7.2
1-acetylchloramphenicol	15	41	88	3.7×10^5	149	1-propionyl-3-iodochloramphenicol	29

[a] Kinetic parameters were determined as described under Experimental Procedures with the exception of those where chloramphenicol was the acetyl acceptor (Lewendon et al., 1988). [b] K_i values were determined as described under Experimental Procedures by using the listed competitive inhibitors and chloramphenicol as acetyl acceptor. [c] Relative specificity, the ratio of k_{cat}/K_m for chloramphenicol and that of the alternative acetyl acceptor, provides a convenient estimate of the extent to which the enzyme discriminates against both binding and acetylation of the C1-substituted analogues.

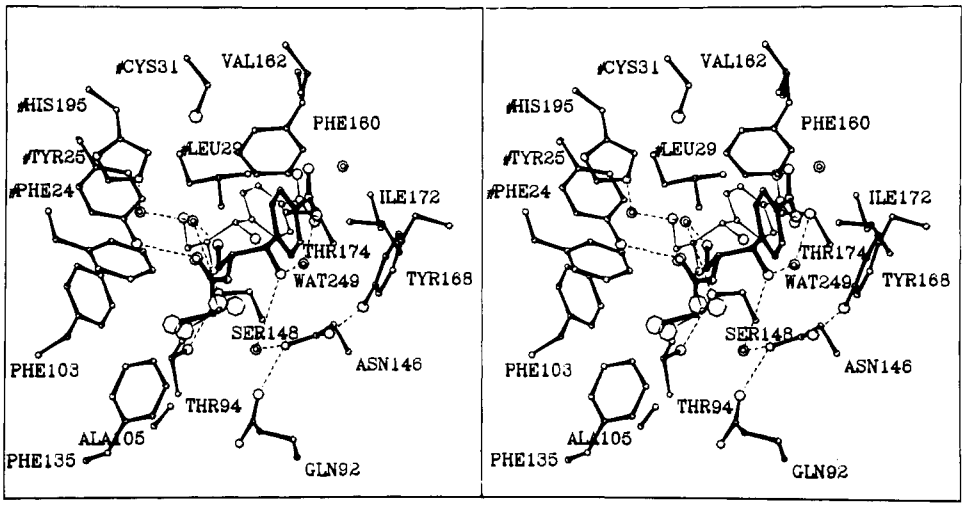


FIGURE 4: Stereoview of residues forming the chloramphenicol binding pocket of Phe-160 CAT. Ordered water molecules are shown as double circles and possible hydrogen bonds are indicated by broken lines. Residue names preceded by # belong to an adjacent subunit of the trimer. The faint chloramphenicol molecule indicates the position occupied in wild-type CAT (cf. Figure 2).

Table III: Kinetic Parameters of Acetyl Transfer to Chloramphenicol, 1-Deoxychloramphenicol, and 1-Acetylchloramphenicol Catalyzed by Gln-160 CAT and Phe-160 CAT[a]

acetyl acceptor (*)	chloramphenicol	1-deoxychloramphenicol	1-acetylchloramphenicol
	Gln-160 CAT		
k_{cat} (s^{-1})	175	51	3.7
K_m* (μM)	116	124	404
K_m acetyl-CoA (μM)	290	319	301
k_{cat}/K_m* (M^{-1} s^{-1})	1.5×10^6	4.1×10^5	9.2×10^3
relative specificity		3.7	163
	Phe-160 CAT		
k_{cat} (s^{-1})	10.5	7	0.18
K_m* (μM)	3.4	19	30
K_m acetyl-CoA (μM)	32	144	66
k_{cat}/K_m* (M^{-1} s^{-1})	3.1×10^6	3.7×10^5	6.0×10^3
relative specificity		8.4	517

[a] Kinetic parameters were determined as described under Experimental Procedures.

the measured affinity for each of the three acetyl acceptors was unchanged, or slightly enhanced, whereas k_{cat} values were reduced between 44- and 83-fold compared with wild type. The k_{cat}/K_m values for transacetylation by Phe-160 CAT of chloramphenicol, 1-deoxychloramphenicol, and 1-acetylchloramphenicol were 3.1×10^6 M^{-1} s^{-1}, 3.7×10^5 M^{-1} s^{-1}, and 6.0×10^3 M^{-1} s^{-1}, respectively. Comparison of k_{cat}/K_m for chloramphenicol and 1-acetylchloramphenicol indicates a 517-fold discrimination against the latter by Phe-160 CAT (cf. 149-fold for wild type). However, the facile explanation that the slightly enhanced discrimination (~3-fold) is a consequence of steric hindrance between the aryl group of Phe-160 and the 1-acetyl substituent of the substrate can be discounted, as discrimination against 1-deoxychloramphenicol, where the volume of the C1 substituent is markedly reduced, also increases.

Structure of Phe-160 CAT. The large fall in rate that accompanies the replacement of Leu-160 by phenylalanine might reflect unexpected displacement of side chains of residues that function in the catalytic cycle and/or binding of the acyl acceptor in a new and less productive conformation. The structure of Phe-160 CAT was determined at 2.0-Å resolution in order to distinguish between these alternatives and to assist interpretation of the kinetic data.

With the exception of the mutated residue, and a small (0.35-Å) shift in the side chain of Phe-158, the structure of Phe-160 CAT is isosteric with that of the wild-type enzyme. The Phe-160 side chain occupies the position predicted by modeling, with the C^{β} and C^{γ} atoms within 0.15 Å of the positions of the same atoms of Leu-160 in the native structure. However, the position of bound chloramphenicol is dramatically different in the two structures (Figure 4). When Leu-160 is replaced by phenylalanine, model building indicates that some movement of either the side chain or the substrate is required to relieve a short contact (2.9 Å) between the C^{ζ} atom of Phe-160 and the 1-hydroxyl of chloramphenicol. As the Phe-160 side chain is tightly packed against neighboring side chains forming the chloramphenicol binding pocket, this would be accommodated most readily by a shift in the position of the substrate by ~0.5 Å. However, in the mutant structure the 1-hydroxyl has actually moved by 2.0 Å. Two features of the chloramphenicol binding site may account for this unexpectedly large shift. The first of these involves the van der Waals interactions between the phenyl ring of chloramphenicol and the side chains of its binding site. In wild-type CAT, the face of this phenyl ring packs against the side chains

of Leu-160 and Ile-172, while the edges of the ring pack against Cys-31 (of an adjacent subunit of the trimer) and Asn-146. In the mutant structure, the plane of the phenyl ring has rotated 25° relative to its position in the native enzyme, so that the face of the ring now packs solely against the side chain of Ile-172, and the ring edge is able to form an edge-to-face aromatic interaction (Burley & Petsko, 1985; Singh & Thornton, 1985) with the side chain of Phe-160. The dihedral angle for this interaction is 67° and the ring centroid separation is 5 Å, very similar to the mean values observed in small molecules (Burley & Petsko, 1985). While not directly relevant to substrate binding, it is of interest to note that the mutated residue Phe-160 also makes a similar aromatic–aromatic interaction with Phe-158 (dihedral angle 69°, ring centroid separation 5 Å). The second feature of note concerns the environment of the 1-hydroxyl of chloramphenicol. In the wild-type structure this is hydrogen bonded to three water molecules (Wat249, 252, and 360; Figures 2 and 3), which themselves form part of an extensive hydrogen-bonding network. In the Phe-160 mutant structure, Wat252 (the water that also forms a hydrogen bond to the hydroxyl of Thr-174) is lost due to its close proximity to the side chain of Phe-160. The result is a much less polar environment, which may promote the observed shift in position of the 1-hydroxyl by 2 Å toward a more polar location on the opposite face of the binding pocket, with the concomitant loss of Wat360. Wat249 also moves, by 0.9 Å, which results in a strong interaction with the new position of the 1-hydroxyl and the hydroxyl of Thr-174.

The overall conformation of chloramphenicol itself is very similar in wild-type and Phe-160 CAT and closely resembles that in the crystal structure of the free antibiotic (Chatterjee et al., 1979). Nonetheless, the orientations of chloramphenicol within its binding sites in the two proteins are quite different. The most significant kinetic consequence of the change in orientation of the substrate in Phe-160 CAT relates to the interaction between the 3-hydroxyl of chloramphenicol and $N^{\epsilon 2}$ of His-195. In wild-type CAT the catalytic imidazole nitrogen is 2.8 Å from the 3-hydroxyl oxygen of the substrate (Leslie, 1990) and 3.3 Å from the same atom of the modeled tetrahedral intermediate (P. C. E. Moody and A. G. W. Leslie, unpublished results) and is critically disposed to abstract a proton from the 3-hydroxyl as the initial event in the catalytic cycle. In the binary complex of chloramphenicol and Phe-160 CAT these distances are increased by a further 1.9 Å (and can be reduced by only ~0.5 Å by adjusting the torsion angles of the 3-hydroxyl), so that the observed conformation is most unlikely to be active. Such a conclusion is supported by studies of the Glu-165 → Asp mutant of triosephosphate isomerase (Hermes et al., 1990), where an increase of 1 Å in the distance between the catalytic base and its target proton results in a 1000-fold decrease in activity. Furthermore, the precise van der Waals contacts between the new position of the aryl group of chloramphenicol and the side chains of Phe-160 and Ile-172 in the mutant (Figure 4) preclude the binding of substrate in a conformation approaching that which occurs with wild-type CAT unless the 1-hydroxyl group is displaced from its new, polar environment. An additional conformation of bound chloramphenicol must therefore be invoked to account for the observed activity of Phe-160 CAT.

Conclusions

The structure of the binary complex of CAT_{III} and chloramphenicol reveals a cavity adjacent to the 1-hydroxyl of the substrate, which might accommodate the acetyl group of 1-acetylchloramphenicol without significantly affecting the mode of binding of the substrate. However, substitution by site-directed mutagenesis of the conserved residue Leu-160, the side chain of which forms part of the surface of the cavity, does not result in significantly enhanced discrimination against the acetylation of the 1-acetyl analogue of the substrate. In addition, both 1-acetyl-3-iodochloramphenicol and 1-propionyl-3-iodochloramphenicol are potent competitive inhibitors of wild-type CAT. These results suggest that the 1-acetyl substituents of the analogues may not bind to the active site in close proximity to $C^{\delta 1}$ of Leu-160 as does the 1-hydroxyl of chloramphenicol. The low rate of turnover of 1-acetylchloramphenicol by the wild-type enzyme is also consistent with the concept of a different, and less productive, mode of binding for the acetylated substrate. We are currently employing NMR techniques to identify residues in close proximity to the methyl groups of [2-^{13}C]-diacetylchloramphenicol to define more precisely the binding site for the 1-acetyl substituent.

The structure of the binary complex of chloramphenicol and Phe-160 CAT reveals an alternative, but probably nonproductive, mode of substrate binding. The fact that Phe-160 CAT is able to acetylate not only chloramphenicol but also its 1-deoxy and 1-acetyl derivatives clearly implies an additional (productive) binding mode for these substrates. Furthermore, as the structure of Phe-160 CAT is isosteric with that of the wild-type enzyme (with the exception of the Phe-160 residue) it is likely that this mechanism, albeit less efficient, is also available to wild-type CAT.

Overall, the results of the present study emphasise a remarkable plasticity in the active site of CAT, manifest by its ability to tolerate disruptive mutations and to accommodate alternative substrates. In this connection it may be useful to recall that the wild-type CAT_{III} is the most highly evolved ($k_{cat}/K_m = 5.2 \times 10^7$ M^{-1} s^{-1}) enzyme of the CAT family (Albery & Knowles, 1976) and that even a 100-fold fall in the specificity constant, largely at the expense of k_{cat}, would still yield an enzyme capable of conferring the phenotype of chloramphenicol resistance on its host. In fact, the specific activity of Phe-160 CAT (19.5 units mg^{-1}), although 40-fold reduced compared with wild-type CAT_{III}, probably exceeds that of some naturally occurring CAT variants that are able to confer chloramphenicol resistance in vivo (Zaidenzaig et al., 1979).

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

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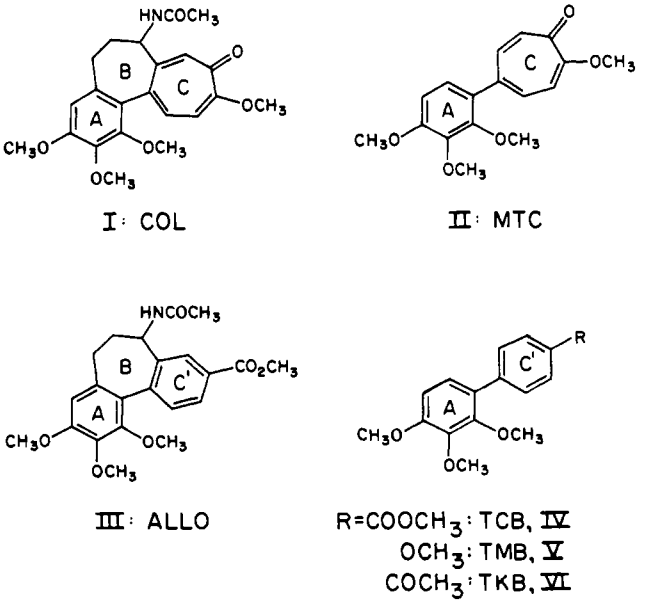
ABSTRACT: The roles of the oxygens in ring C of colchicine in its binding to tubulin were probed by a study of the interactions of two allocolchicine biphenyl analogues, 2,3,4,4′-tetramethoxy-1,1′-biphenyl (TMB) and 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl (TKB), the first one containing a methoxy group 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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