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Structure-Function Studies with Derivatives of 6-Benzyl-1,3-benzodioxole, a New Class of Synthetic Compounds Which Inhibit Tubulin Polymerization and Mitosis

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SUMMARY

A new class of synthetic antineoplastic compounds, derivatives of 6-benzyl-1,3-benzodioxole, has significant antimitotic activity. These compounds inhibit microtubule assembly and are competitive inhibitors of the binding of colchicine to tubulin. Both their structure and their partial inhibition of tubulin-dependent GTP hydrolysis indicate that they are most comparable to podophyllotoxin of all known antimitotic drugs. Maximum activity required an intact dioxole ring, a methoxy or ethoxy substituent at position 5, and, on the benzyl moiety at position 6, a *para*-methoxy group. Additional methoxy groups on the benzyl substituent, to increase the apparent structural similarity to podophyllotoxin, resulted in major reduction of the antitubulin activity of these drugs.

INTRODUCTION

A number of derivatives of 6-benzyl-1,3-benzodioxole, originally synthesized as potential insect sterilants (1, 2), have had significant cytotoxic activity in the drug-screening surveys used by the National Cancer Institute to identify potential new antineoplastic agents.[1] Our laboratory has been examining cytotoxic drugs of unknown mechanism of action for effects on tubulin-dependent GTP hydrolysis (3) to select those whose activity probably results from inhibition of mitosis. In initial studies, the cytocidal benzyl-benzodioxole derivatives partially inhibited this reaction, thus most closely resembling podophyllotoxin of all the antimitotic drugs so far evaluated by this assay (3–5). Further, the benzyl-benzodioxole compounds have clear structural analogies to both podophyllotoxin and steganacin (see Fig. 1). As will be described in this report, the antimitotic and antitubulin effects predicted by the GTPase assay for these new agents were confirmed. In addition, we have demonstrated that the benzyl-benzodioxole derivatives, like podophyllotoxin and several other antimitotic drugs (6–10), are competitive inhibitors of the binding of colchicine to tubulin.

In this last regard, these new agents are of particular interest. A large number of them have already been prepared (1, 2),[2] and their synthesis and characterization are relatively facile. Consequently, they are potentially of great value in structure-function studies of the colchicine/podophyllotoxin-binding site. We have examined the currently available compounds to define essential features for their interaction with tubulin.

MATERIALS AND METHODS

Materials. Purified calf brain tubulin and heat-treated microtubule-associated proteins were prepared as described previously (11, 12). Vinblastine, nonradioactive colchicine, GTP (repurified by gradient chromatography on DEAE-Sephadex A-25), and 2-(*N*-morpholino)ethanesulfonate (free acid, adjusted to pH 6.4 with NaOH) were obtained from Sigma; podophyllotoxin was from Aldrich; [*ring* A-4-^{3}H] colchicine and $^{32}P_i$ were from Amersham; and monosodium glutamate (adjusted to pH 6.6 with HCl) was from Grand Island Biological Corp. The 6-benzyl-1,3-benzodioxole derivatives were prepared as described elsewhere (1, 2).[2] It should be noted that, in most of these agents, the bridge carbon between the B and C rings is an optically active center. The preparations used here were mixtures of the isomers. All drugs were dissolved in dimethyl sulfoxide, and control reaction mixtures contained equivalent amounts of the solvent. Steganacin was a generous gift of Dr. J.-P. Robin. Maytansine was provided by the Natural Products Branch of the National Cancer Institute. The method of Walseth and Johnson (13) was used to prepare [γ-^{32}P]GTP.

Methods. In vitro polymerization of tubulin was followed turbidimetrically (14) in a Gilford model 250 recording spectrophotometer equipped with a Gilford Thermoset electronic temperature controller.

[1] Among those most extensively tested in murine tumors were NSC 350102 and 321567 (see Fig. 4 for structural details). Both compounds were effective against P388 leukemia *in vivo* (tumor cells and drug injected intraperitoneally), but negative results were obtained with the B16 melanoma, L1210 leukemia, the M5076 reticulum cell sarcoma, and a human mammary carcinoma xenograft in nude mice. In addition, negative results were obtained with NSC 321567 in the Lewis lung carcinoma, CDF_1 mammary carcinoma, and colon 38 carcinoma.

[2] L. Jurd, unpublished observations, manuscript in preparation.

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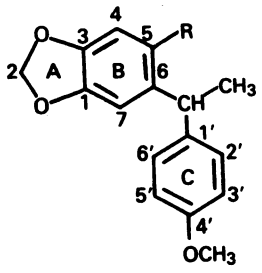
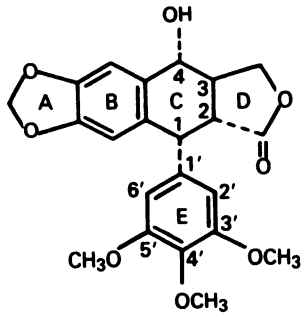
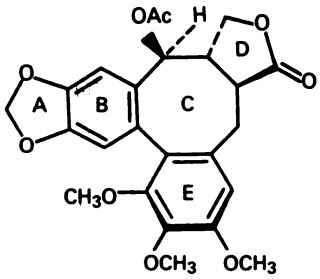






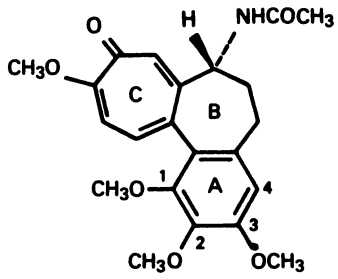


FIG. 1. *Structures of 6-benzyl-1,3-benzodioxole derivatives, podophyllotoxin, steganacin, and colchicine*

After baselines were established at 0°, reaction temperature was set at 37° at zero time (reaction mixtures were at 34° at 70 sec, and equilibrated at 37° at about 2 min). Colchicine binding was measured by the DEAE-cellulose filter assay (15) essentially as described elsewhere (9). L1210 cells were used to examine the effects of drugs on the mitotic index and in preliminary cytotoxicity studies, as described by Wolpert-DeFilippes *et al.* (16). GTP hydrolysis was measured by following the formation of $^{32}P_i$ from [γ-^{32}P]GTP, using thin layer chromatography on polyethyleneimine-cellulose and autoradiography (11, 12).

RESULTS

Antitubulin and antimitotic properties of 6-benzyl-1,3-benzodioxole derivatives. Two of the most potent benzyl-benzodioxole derivatives, NSC 321567 and NSC 350102 (see Fig. 4 for structural details), initially chosen on the basis of their cytotoxicity, were examined for their effects in several tubulin-dependent reactions. As noted above, these compounds were observed to partially inhibit tubulin-dependent GTP hydrolysis in our screening assay (3). This assay relies on 1.0 M glutamate to activate the GTPase activity of tubulin (5, 11). Table 1, Experiment I, presents an experiment in which the effects of these two drugs on GTP hydrolysis were compared to those of several known antimitotic drugs. Podophyllotoxin, steganacin (7), and colchicine (structures presented in Fig. 1) all bind at the same site on tubulin and contain a trimethoxybenzene ring, while maytansine and vinblastine bind at a different site on the protein (6, 17). In 1.0 M glutamate, colchicine (5; cf. Ref. 4) enhances GTP hydrolysis, while maytansine (5) and vinblastine (5; cf. Ref. 4) totally inhibit the reaction. Podophyllotoxin only partially inhibits hydrolysis, however, and, in their effects on this reaction, NSC 321567 and NSC 350102 more closely resemble podophyllotoxin than any other drug thus far examined. The effect of steganacin differed little from that of colchicine, enhancing rather than inhibiting GTP hydrolysis, despite the drug's structural similarity to podophyllotoxin. (It should also be noted that no polymerization reaction occurs with the drugs at 0.1 mM in 1.0 M glutamate.[3])

At low ionic strengths, tubulin has little GTPase activity, but in the presence of colchicine a relatively slow hydrolytic reaction is induced (3, 4, 9). Steganacin shares this property with colchicine, while podophyllotoxin (cf. Ref. 4) and the benzyl-benzodioxole derivatives have no effect, even inhibiting the minimal residual GTPase re-

[3] J. K. Batra and E. Hamel, unpublished observations.

TABLE 1

Effects of benzyl-benzodioxole derivatives, podophyllotoxin, and other antimitotic agents on tubulin-dependent GTP hydrolysis

Each 50-μl reaction mixture contained 1.0 mg/ml of purified tubulin, 0.1 mM [γ-^{32}P]GTP, the indicated drug at 0.1 mM, and either 1.0 M glutamate (Exp. I), 0.1 M glutamate (Exp. II), or 0.1 M 2-(*N*-morpholino)ethanesulfonate (pH 6.4), 0.5 mM $MgCl_2$, and 0.5 mg/ml of heat-treated microtubule-associated proteins (MAPs). Incubation was for 20 min at 37°. Data are expressed as nanomoles of P_i formed per ml of reaction mixture. Duplicate determinations were within 10% of each other. In Exps. I and II, the dimethyl sulfoxide concentration was 10% (v/v), and in Exp. III was 1% (v/v).

Drug added	Exp. I (1.0 M glutamate)	Exp. II (0.1 M glutamate)	Exp. III (with MAPs)
	nmol P_i formed		
None	11.9	1.1	20.4
NSC 321567	6.9	0.7	1.2
NSC 350102	7.5	0.8	1.5
Podophyllotoxin	3.5	0.4	0.8
Colchicine	33.0	10.5	21.2
Steganacin	26.6	8.0	15.2
Vinblastine	0	0	0
Maytansine	0	0	0
None (no dimethyl sulfoxide)			19.2
None (no MAPs)			0.6
None (neither MAPs nor dimethyl sulfoxide)			0.4

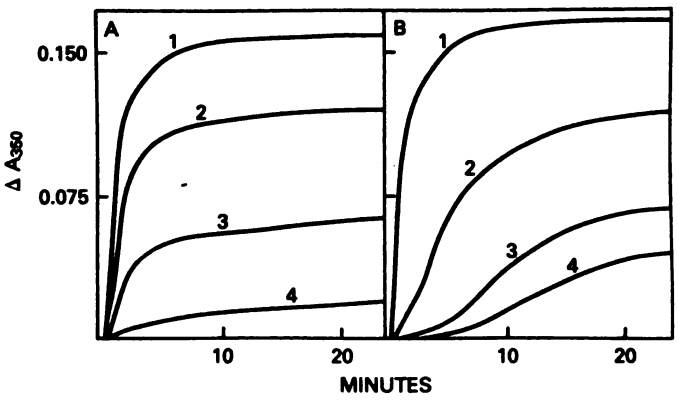


FIG. 2. *Inhibition of microtubule assembly by podophyllotoxin and NSC 321567*

Each 0.25-ml reaction mixture contained 0.75 mg/ml of purified tubulin, 0.38 mg/ml of heat-treated microtubule-associated proteins, 0.1 M 2-(N-morpholino)ethanesulfonate (pH 6.4), 0.5 mM $MgCl_2$, 1.0 mM GTP, 2% (v/v) dimethyl sulfoxide, and drugs as indicated. A, effect of podophyllotoxin. *Curve 1*, none; *curve 2*, 2.5 μM; *curve 3*, 5 μM; and *curve 4*, 7.5 μM. B, effect of NSC 321567. *Curve 1*, none; *curve 2*, 5 μM; *curve 3*, 7.5 μM; and *curve 4*, 10 μM.

action (Table 1, Experiment II). Maytansine and vinblastine were again totally inhibitory.

When heat-treated microtubule-associated proteins (which have no GTPase activity) were added at low ionic strengths, tubulin-dependent GTP hydrolysis was again induced (Table 1, Experiment III). As in 1.0 M glutamate, no polymerization occurred with the drugs at 0.1 mM.[3] Again, the effects of the benzyl-benzodioxole derivatives more closely resembled that of podophyllotoxin, and the

TABLE 2

Effect of NSC 321567 and NSC 350102 on the mitotic index of L1210 cells

At least 400 cells were counted at each drug concentration. In the control experiment, the mitotic index was 2.9.

Drug added	NSC 321567	NSC 350102
μM	*mitotic index*	
1	7.9	7.1
3	36.1	29.1
10	77.4	84.0

effect of steganacin was more comparable to that of colchicine. The benzyl-benzodioxole derivatives and podophyllotoxin were 93–96% inhibitory, while steganacin was only 25% inhibitory and colchicine slightly stimulated the reaction. Maytansine and vinblastine were totally inhibitory.

Fig. 2 presents a study comparing the inhibitory effects of podophyllotoxin (panel A) to that of NSC 321567 (panel B) on microtubule assembly in a reconstituted system of purified tubulin and heat-treated microtubule-associated proteins. There were only minor differences between the two drugs. The benzyl-benzodioxole derivatives thus belong to the large group of drugs which inhibit tubulin polymerization.

Thus far, most antimitotic drugs have significant antitubulin activity *in vitro* and vice versa. We therefore examined NSC 321567 and NSC 350102 for antimitotic effects on L1210 cells in culture. In preliminary cytotoxicity studies, the LD_{50} for NSC 321567 was found to be about 0.5 μM. The data presented in Table 2 demonstrate that these active benzyl-benzodioxole derivatives are antimitotic agents, with cells accumulating in metaphase arrest at drug concentrations as low as 1.0 μM. Both drugs exhibited similar effects at all concentrations, and at 10 μM about 80% of the cells displayed mitotic figures.

All active derivatives were found to inhibit the binding of colchicine to tubulin.[3] NSC 350102 was selected for more detailed studies, presented as modified Dixon plots in Fig. 3A and Lineweaver-Burk plots in Fig. 3B. NSC 350102 proved to be a competitive inhibitor of the binding of colchicine to tubulin with an apparent K_i of 0.62 μM.

Structure-function studies with benzyl-benzodioxole derivatives. In the studies to be presented below, we have compared the inhibitory effects of benzyl-benzodioxole derivatives on the glutamate-induced polymerization of purified tubulin (11) to determine their relative activity. For these studies, we have defined the ID_{50} as the concentration of drug which reduced the turbidity reading at 20 min by 50% (7, 18). We have used glutamate-induced polymerization, even though sheets of protofilaments rather than microtubules are formed (3), for two reasons. First, this reaction eliminates the possibility that microtubule-associated proteins affect the drug-tubulin interaction. Second, in initial experiments with some active derivatives, we found that with microtubule-associated proteins there was a significant temperature-dependent, cold-irreversible aggregation reaction which altered the apparent ID_{50} value. For comparison, in this system, the ID_{50} value for podophyllotoxin was 0.5–1.0

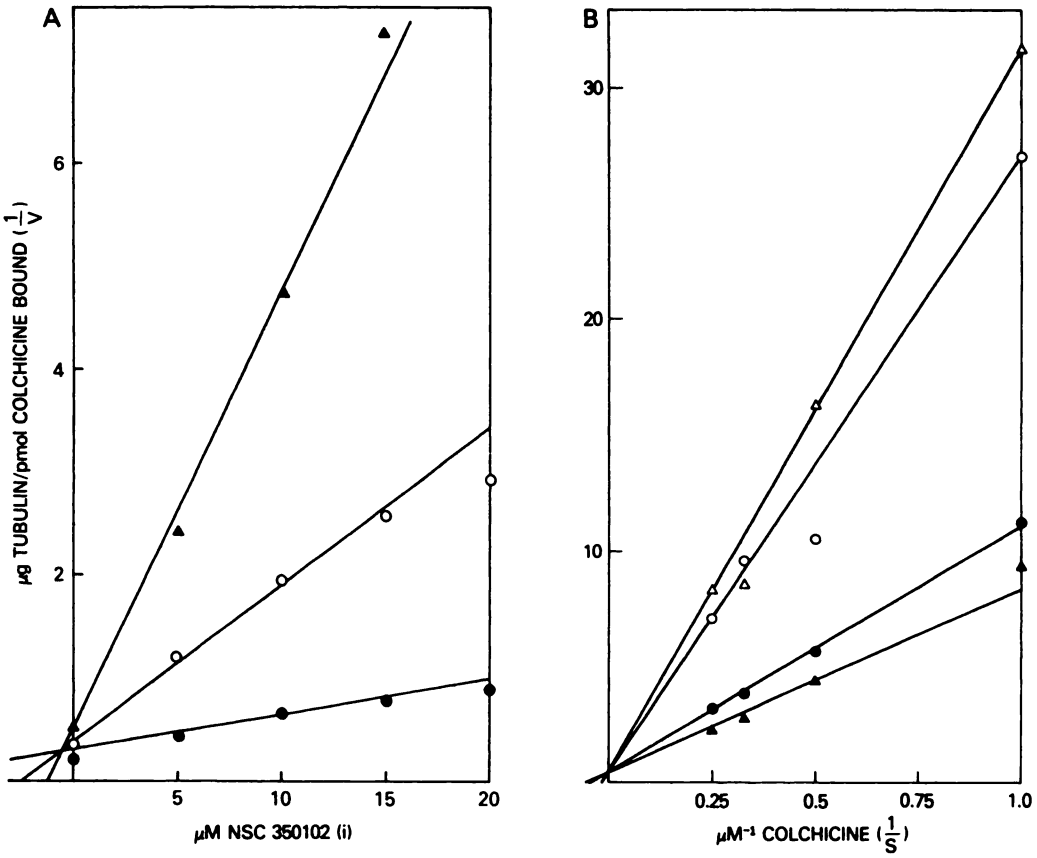


FIG. 3. *Competitive inhibition of colchicine binding to tubulin by NSC 350102*

A, analysis by the Dixon method. The concentrations of [*ring* A-4-^{3}H]colchicine were as follows: ▲, 1 μM; ○, 2 μM; and ●, 4 μM. Reaction mixtures contained NSC 350102 at the indicated concentrations. Incubation was for 60 min at 37°. B, analysis by the Lineweaver-Burk method. The concentrations of NSC 350102 were as follows: ▲, none; ●, 1 μM; ○, 2 μM; and △, 3 μM. Incubation was for 2 min at 37°. Reaction mixtures contained 0.4 mg/ml of tubulin, [*ring*-A-4-^{3}H]colchicine at the indicated concentrations, 2 mM $MgCl_2$, and other components as described previously (9). Filtration was through a stack of three DEAE-cellulose filters, instead of two, because of the 4-fold higher tubulin concentration used in this experiment.

μM and for steganacin was 5 μM. All compounds which inhibit glutamate-induced polymerization also inhibit the microtubule-associated protein-dependent reaction, as shown above in Fig. 2 for NSC 321567.

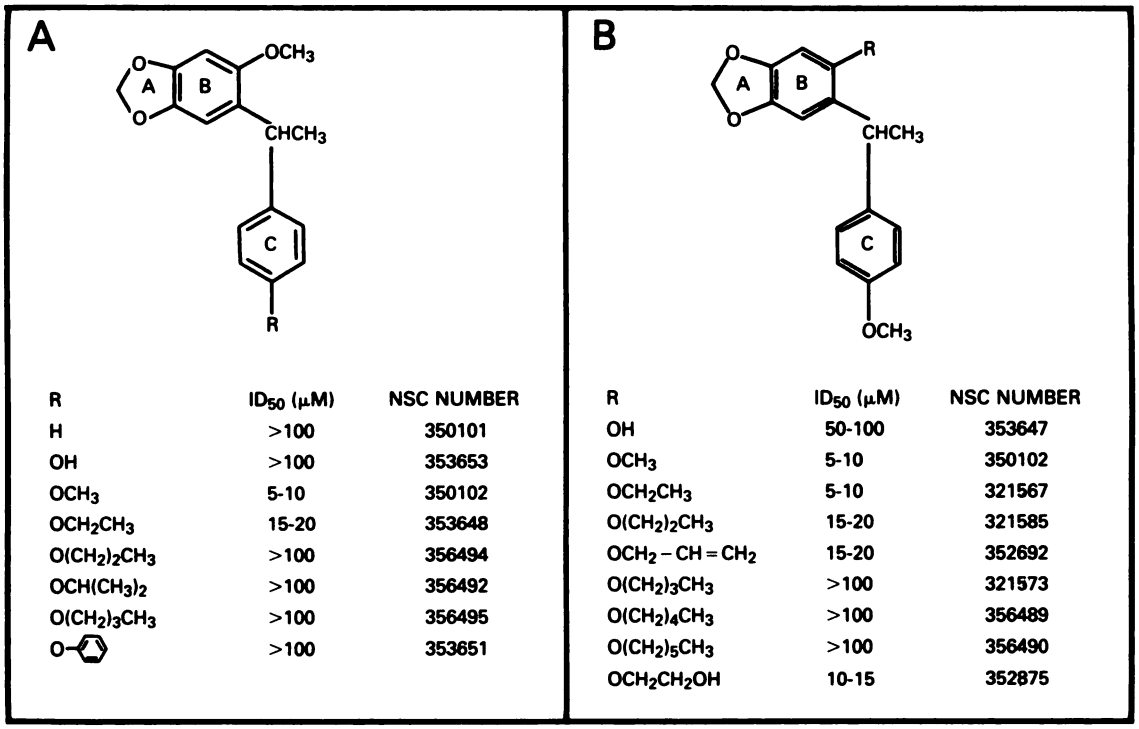
One of the most stringent requirements for antitubulin activity in the structure of the benzyl-benzodioxole derivatives was the substituent at the 4′-position of the C ring (Fig. 4A). Of the available compounds, only those with a methoxy (NSC 350102) or ethoxy (NSC 353648) group had significant inhibitory activity, with the former compound being at least twice as active as the latter. Derivatives with either bulkier substitutents or none at all were inert or had only minimal inhibitory effects. Fig. 5 presents typical reactions with both active (panel A) and relatively inactive (panel B) members of this series. It should be pointed out that the benzyl-benzodioxole derivatives inhibit not only the extent of polymerization, but also its rate. If rate rather than extent were used to define the ID_{50}, the values obtained would be considerably lower. For example, the maximum rate observed with 10 μM NSC 353648 (Fig. 5A, *curve 2*) was only 25% of that of the uninhibited reaction, although the turbidity reading at 20 min was over 70% of the control value. Similarly, with 100 μM NSC 356494 (Fig. 5B, *curve 3*), the extent of polymerization was almost identical to that of the control reaction, but the maximal rate was only 45% of the control value.[4]

Three derivatives were available with the methoxy group at the 2′-position, and all were inert at 100 μM.[5] A single derivative was available with the methoxy group at the 3′-position (NSC 353670, a hydroxyl at the 4′-position, an ethoxy group at position 5). This compound had an ID_{50} of 50–100 μM.

At the 5-position of ring B (Fig. 4B), maximum activity was again observed with methoxy (NSC 350102) and ethoxy (NSC 321567) substituents. Addition of a third carbon, either as propyloxy (NSC 321585) or allyloxy (NSC 352692) reduced activity by about 50%, while compounds with still longer substituents were essentially inert. A hydroxy substituent at the 5-position resulted in a compound with sharply reduced inhibitory activity

[4] All glutamate-induced polymerization reactions were also evaluated for cold reversibility (reactions without drug were over 90% cold-reversible), and the relative activity of compounds was the same whether total turbidity plateau, cold-reversible reaction, or rates were compared. In the glutamate-induced reaction, both sedimentable protein and turbidity plateau are linear functions of the tubulin concentration in the reaction mixture within the range studied here.

[5] The substituents at the 5-position were methoxy (NSC 357746), ethoxy (NSC 357747), and allyloxy (NSC 359297).



R	ID_{50} (μM)	NSC NUMBER
H	>100	350101
OH	>100	353653
OCH_3	5-10	350102
OCH_2CH_3	15-20	353648
$O(CH_2)_2CH_3$	>100	356494
$OCH(CH_3)_2$	>100	356492
$O(CH_2)_3CH_3$	>100	356495
O-⬡	>100	353651

R	ID_{50} (μM)	NSC NUMBER
OH	50-100	353647
OCH_3	5-10	350102
OCH_2CH_3	5-10	321567
$O(CH_2)_2CH_3$	15-20	321585
$OCH_2-CH=CH_2$	15-20	352692
$O(CH_2)_3CH_3$	>100	321573
$O(CH_2)_4CH_3$	>100	356489
$O(CH_2)_5CH_3$	>100	356490
OCH_2CH_2OH	10-15	352875

FIG. 4. *Effect of substituents at the 4′- and 5-positions on the inhibition of tubulin polymerization by benzyl-benzodioxole derivatives*
Each 0.25-ml reaction mixture contained 1.0 mg/ml of purified tubulin, 1.0 M glutamate, 1.0 mM $MgCl_2$, 0.1 mM GTP, 2% (v/v) dimethyl sulfoxide, and the indicated drugs at various concentrations. ID_{50} is defined as described in the text. When a range is given, this indicates that inhibition was less than 50% at the lower concentration and over 50% at the higher concentration. All experiments were performed at least three times at drug concentrations which defined an ID_{50} value. A, effect of substituents at the 4′-position. B, effect of substituents at the 5-position.

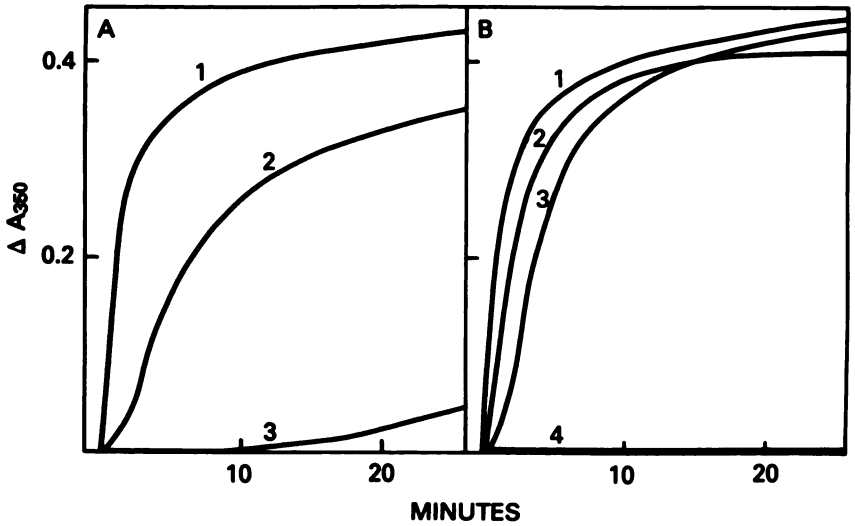
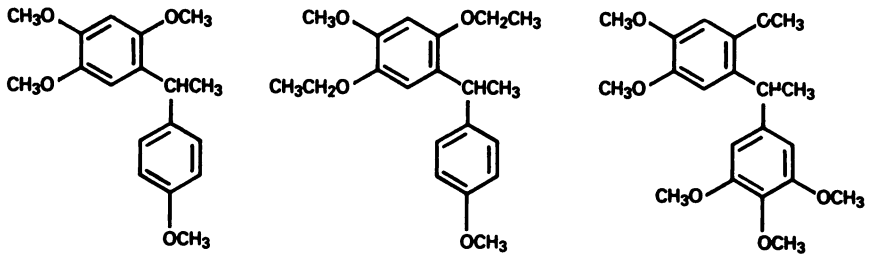


FIG. 5. *Inhibition of tubulin polymerization by benzyl-benzodioxole derivatives modified at the 4′-position (see Fig. 4 for structures)*
Experimental conditions were as described in the legend of Fig. 4, except for drug concentrations as indicated below. A, effect of lower drug concentrations. *Curve 1*, none; *curve 2*, 10 μM NSC 353648; and *curve 3*, 10 μM NSC 350102. B, effect of higher drug concentrations. *Curve 1*, none; *curve 2*, 100 μM NSC 356494; *curve 3*, 100 μM NSC 353653; and *curve 4*, 100 μM NSC 353648.

(NSC 353647); but this did not result from the hydroxyl group itself, as the derivative with a hydroxyethoxy group at position 5 had good activity (NSC 352875).

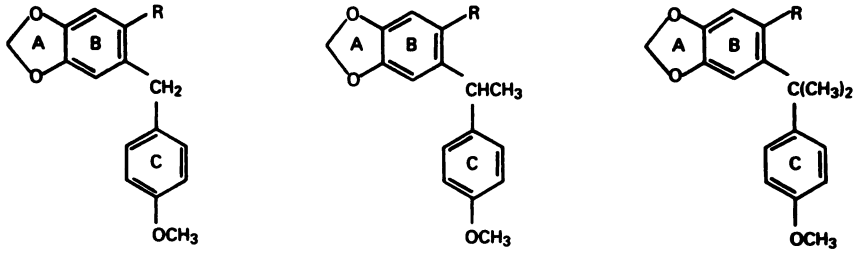
Only a few analogues are presently available with a disrupted A ring. These are shown in Fig. 6. Compound I had an ID_{50} of 50–100 μM, while II and III were noninhibitory at 100 μM.

A limited number of compounds are presently available with modifications at the one carbon bridge connecting the B and C rings. These modifications all result in optically symmetrical compounds, representing variants of the racemic NSC 321567 and NSC 350102, with either a methylene bridge or a dimethyl-substituted methylene bridge between the two rings (Fig. 7). These changes at the bridge carbon had only minor effects on the inhibitory activity of the benzyl-benzodioxole derivatives. One



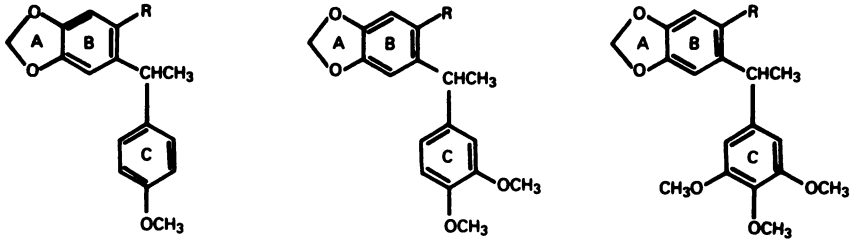
I II III

FIG. 6. *Structures of inactive compounds with a disrupted dioxole ring*



R	ID_{50} (μM) (NSC NUMBER)		
OCH_3	20-25 (269130)	5-10 (350102)	5 (364720)
OCH_2CH_3	5-10 (321584)	5-10 (321567)	10-15 (364721)

FIG. 7. *Effect of modifications at the bridge carbon on the inhibition of tubulin polymerization by benzyl-benzodioxole derivatives* Conditions were as described in the legend of Fig. 4, except for the drugs added, as indicated.



R	ID_{50} (μM) (NSC NUMBER)		
OCH_3	5-10 (350102)	>100 (353649)	>100 (352683)
OCH_2CH_3	5-10 (321567)	20-25 (352687)	>100 (352682)
$O(CH_2)_2CH_3$	15-20 (321585)	—	>100 (353438)
$OCH_2-CH=CH_2$	15-20 (352692)	>100 (355069)	—
OCH_2CH_2OH	10-15 (352875)	>100 (353660)	>100 (352876)

FIG. 8. *Loss of inhibitory activity of benzyl-benzodioxole derivatives on tubulin polymerization caused by additional methoxy substituents on the C ring*

Conditions were as described in the legend of Fig. 4, except for the drugs added, as indicated.

of these compounds (NSC 364720), however, is most active of all those we have examined, provided the two isomers of NSC 321567 and NSC 350102 are equally active.

One more group of benzyl-benzodioxole derivatives were of particular interest, those with one or two additional methoxy groups attached to the C ring, for such additions would appear to increase the structural analogy between this class of agents and podophyllotoxin. These modifications were available in five derivatives with good

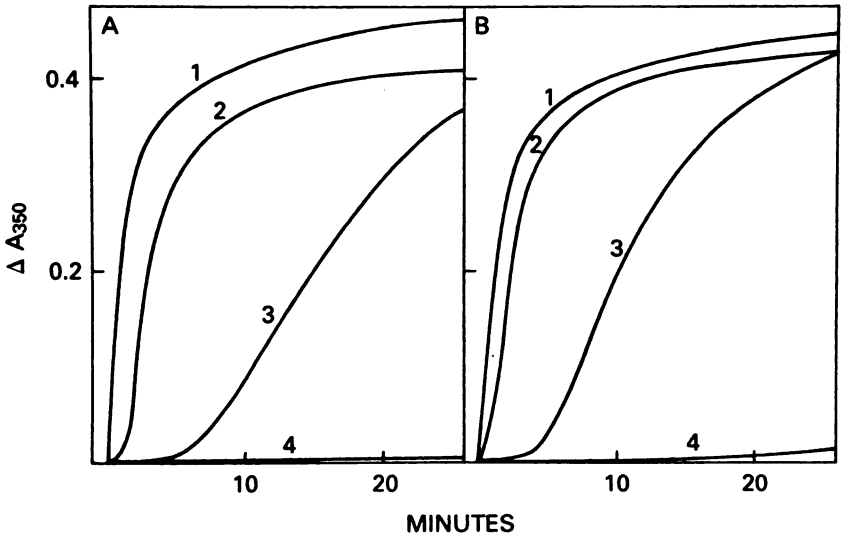
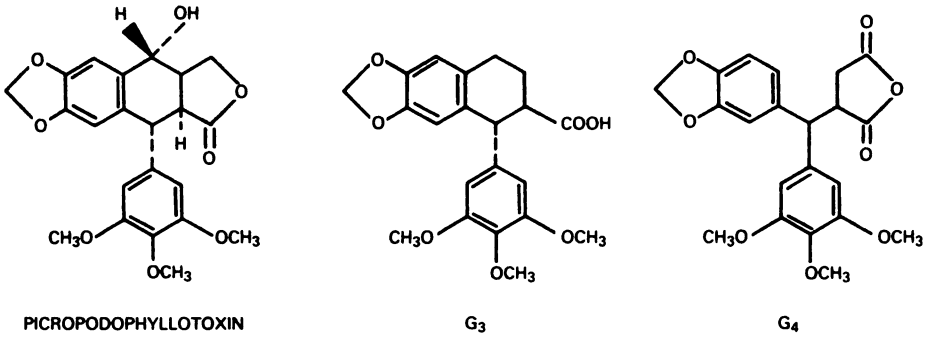


FIG. 9. *Inhibition of tubulin polymerization by benzyl-benzodioxole derivatives modified in the C ring (see Fig. 8 for structures)*
Experimental conditions were as described in the legend of Fig. 4, except for drug concentrations as indicated. A, effect of modified derivatives with a methoxy group at position 5. *Curve 1*, none; *curve 2*, 100 μM NSC 352683; *curve 3*, 100 μM NSC 353649; and *curve 4*, 100 μM NSC 350102. B, effect of modified derivatives with an ethoxy group at position 5. *Curve 1*, none; *curve 2*, 100 μM NSC 352682; *curve 3*, 20 μM NSC 352687; and *curve 4*, 20 μM NSC 321567.



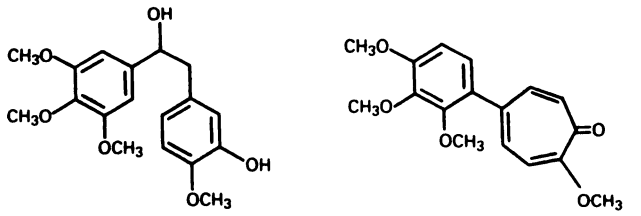


FIG. 10. *Structures of picropodophyllotoxin, the podophyllotoxin analogues G3 and G4, combretastatin, and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone*

activity with a single methoxy group (Fig. 8). Surprisingly, in every case, additional methoxy groups substantially reduced the inhibitory effects of benzyl-benzodioxole derivatives on tubulin polymerization. Fig. 9 presents typical polymerization curves with the NSC 350102 (panel A) and NSC 321567 (panel B) series. In both cases, the derivative with a trimethoxybenzene ring was less active than that with a dimethoxybenzene ring. (In the third complete series, that based on NSC 352875, the trimethoxy compound was slightly more inhibitory than the dimethoxy analogue.) These derivatives with additional methoxy groups also had little or no effect on the binding of colchicine to tubulin and on tubulin-dependent GTP hydrolysis.[3]

DISCUSSION

The studies presented here demonstrate that the most active 6-benzyl-1,3-benzodioxole derivatives represent a

new class of synthetic antimitotic compounds. These drugs inhibit tubulin polymerization both with and without microtubule-associated proteins and are competitive inhibitors of the binding of colchicine to tubulin. Their structure and their partial inhibition of tubulin-dependent GTP hydrolysis indicate that they are most comparable to podophyllotoxin of the previously described antimitotic agents.

Currently available derivatives demonstrate that the dioxole ring must be intact and that the 5-position must bear a substituent with one to three carbon atoms (optimum: a methoxy or ethoxy group). Larger substitutents may limit free rotation of the two ring systems, and hence the ability of the molecules to adopt a conformation able to bind efficiently to tubulin. It is also possible that the substituent at position 5 together with the bridge carbon between the B and C rings represent a structure analogous to the C ring of podophyllotoxin. Further, the 4′-position must bear a substituent with one or two carbon atoms (optimum: a methoxy group), and placement of this substituent at either the 2′- or 3′-position produced compounds with little or no activity.

Particularly surprising was the major reduction in activity observed when either one or two additional methoxy groups were placed on the C ring of active benzyl-benzodioxole derivatives. Thus, the compounds superficially most structurally comparable to podophyllotoxin were the least active. A number of observations in the literature may help to explain this unexpected finding.

Although there are no reported studies with demethoxylated analogues of colchicine or podophyllotoxin, several workers have examined demethylated analogues of the two drugs (18–21). The activity of podophyllotoxin, epipodophyllotoxin, and deoxypodophyllotoxin was unaltered or even enhanced by removal of the methyl group at the 4′-position (18, 19). Colchicine, however, was made less active by demethylation at any position of the trimethoxybenzene ring, and removal of two methyl groups resulted in still further reduction in activity (20, 21). Nevertheless, demethylation at position 1 resulted in more significant loss of activity than demethylation at either position 2 or 3. [The order of activity was reported as colchicine > 3-demethylcolchicine > 2-demethylcolchicine > 1-demethylcolchicine > 2,3-didemethylcolchicine > 1,2-didemethylcolchicine (20, 21).] Consistent with these analogue studies of podophyllotoxin and colchicine was an earlier suggestion, based on examination of molecular models, that the 5′-methoxy group of podophyllotoxin corresponded to the 1-methoxy group of colchicine (22).

Molecular models have also indicated that in podophyllotoxin the E ring is relatively restricted in its orientation and not able to rotate freely (23). Picropodophyllotoxin, 50-fold less active than podophyllotoxin as an inhibitor of tubulin polymerization (18), has a trimethoxybenzene ring with free rotation (23) as a consequence of reversal of configuration at position 2 (see Fig. 10). Two additional derivatives of podophyllotoxin in which the trimethoxybenzene ring has free rotation, termed G3 and G4 (see Fig. 10), have no antitubulin activity (18).

These findings suggest that the potent antitubulin effect of podophyllotoxin derives at least in part from the relative immobility of the E ring. In the 6-benzyl-1,3-benzodioxole derivatives the free rotation of the C ring results in compounds with little or no activity if this ring bears three methoxy groups and maximal activity if it bears a single methoxy group at position 4′. As 4′-demethylpodophyllotoxin is highly active (18, 19), it is unlikely that the 4′-methoxy group of the benzyl-benzodioxole derivatives corresponds to the 4′-methoxy group of podophyllotoxin. The analogy in the two drugs probably lies between the 5′-methoxy group of podophyllotoxin and the required 4′-methoxy group of the benzyl-benzodioxole derivatives.

At the same time, however, we must note that a trimethoxy group with free rotation does not always lead to poor antitubulin activity. Both 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (8, 10, 24) and combretastatin (10) (structures shown in Fig. 10) are effective inhibitors of *in vitro* tubulin polymerization and the binding of colchicine to tubulin, and both these drugs are similar to colchicine in their effects on tubulin-dependent GTP hydrolysis (10). The second ring in these compounds, or perhaps the length of the bridge connecting the two rings, must also play a significant role in the binding of the trimethoxy benzene ring to tubulin. It is also possible that analogues of these two drugs with fewer methoxy groups would have enhanced antitubulin activity, for neither is as potent as podophyllotoxin in inhibiting tubulin polymerization or the binding of colchicine to tubulin (10).

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