

# Synthesis of Alkoxy-Substituted Diaryl Compounds and Correlation of Ring Separation with Inhibition of Tubulin Polymerization: Differential Enhancement of Inhibitory Effects under Suboptimal Polymerization Reaction Conditions

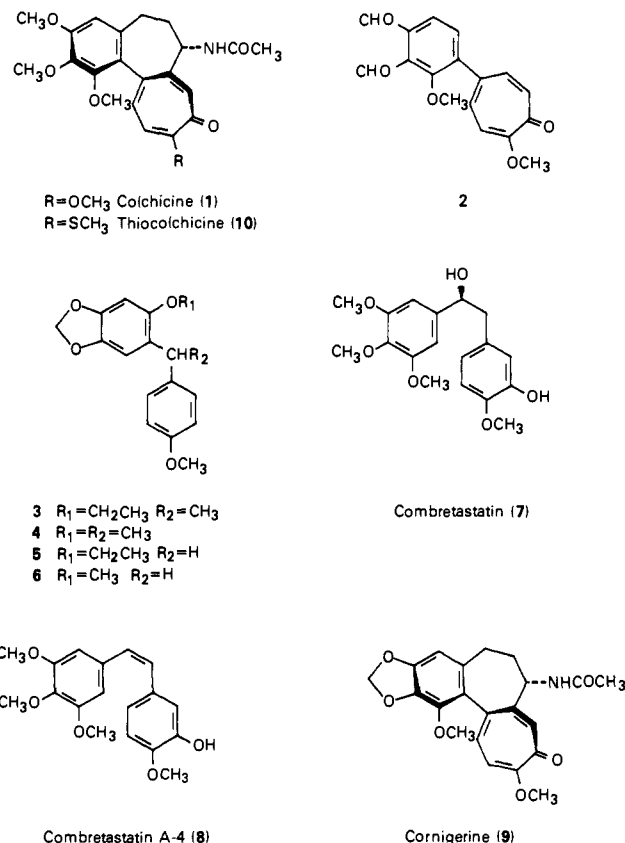
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A number of cytostatic compounds (2-4, 7, and 8), which can be described as "diaryl", inhibit tubulin polymerization, cause cells to accumulate in mitotic arrest, and competitively inhibit the binding of colchicine to tubulin. They differ, however, in the separation of the two aryl moieties. To attempt to understand this variability we prepared a series of analogues modeled on 3 and 4 ("benzodioxole series") and on 7 and 8 ("combretastatin series") which differed only in the number of methylene units (ranging from none to four) separating the aryl moieties. These compounds were evaluated for their effects on tubulin polymerization, colchicine binding, and the growth of L1210 murine leukemia cells. In terms of inhibitory effects on tubulin polymerization, for the combretastatin series there was an optimal separation of the two phenyl rings by a two-carbon bridge (compound 24), with progressively decreasing inhibitory activity when the separation was by one carbon (20), three carbons (25), or four carbons (28) (the biphenyl analogue 16 was inactive). The benzodioxole series, however, did not permit us to generalize this finding, because the least active agents prepared (39 and 40) had a two-carbon bridge, while those with one- (5 and 6) and three-carbon (46 and 47) bridges were nearly equivalent in potency. Submicromolar  $IC_{50}$  values for inhibition of L1210 cell growth were only obtained for compounds 20 ( $IC_{50}$ , 0.2  $\mu$ M), 24 (0.07  $\mu$ M), and 25 (0.4  $\mu$ M). While evaluating the effects of these agents on tubulin polymerization, we noted with the combretastatin series and with several standard agents that apparent potency (in terms of  $IC_{50}$  values) was always lower if the reaction was performed at 30 °C, with 0.25 mM  $MgCl_2$ , than at 37 °C, with 1.0 mM  $MgCl_2$ . This enhancement of  $IC_{50}$  values in the former system as compared with the latter was particularly dramatic for the less active agents (e.g., 28) as compared with the more active (e.g. 24).

The microtubule system of eucaryotic cells represents a potential target for antineoplastic agents.<sup>1</sup> Virtually all drugs which interfere with microtubule structure and function interact with their major component, the dimeric protein tubulin. These agents prevent the normal function of the mitotic spindle, and cells treated with them generally have a tetraploid DNA content and display condensed chromosomes instead of a nucleus (a state referred to as "mitotic arrest"). Examples of useful antitubulin compounds are the well-established vinca alkaloids and the promising new agent taxol. Yet most compounds which interact with tubulin differ in their mechanism of action from these therapeutically useful agents, because they bind in a different region of the tubulin molecule. Although ill-defined at the molecular level, this unexploited (in cancer therapy) region of tubulin is known as the "colchicine site", after the classic drug<sup>1,2</sup> whose tight binding to tubulin led to the original purification of the protein.<sup>3</sup> In general, the molecular structures of compounds which bind in the colchicine site are much simpler than those of taxol and of compounds which bind in the vinca domain. The simplicity of some of these molecules offers promise for the rational design of antitubulin agents.

In particular, a group of compounds which may be loosely described as "diaryl" have been shown to be effective inhibitors of *in vitro* tubulin polymerization and of the binding of radiolabeled colchicine to tubulin. Some examples of such agents are 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone (2),<sup>4</sup> derivatives of benzyl-4,5-benzodioxole (such as 3-6),<sup>5</sup> combretastatin (7),<sup>6-8</sup> and, most potent of all, combretastatin A-4 (8).<sup>9-11</sup> All of these compounds are competitive inhibitors of the binding of colchicine to tubulin, which indicates that they bind at the same site on the protein, and structural analogies with colchicine (1) and the related colchicinoid cornigerine (9)<sup>12,13</sup> are readily apparent in all cases.



A puzzling structural aspect of the diaryl agents, however, is the differing separation of the two aromatic rings

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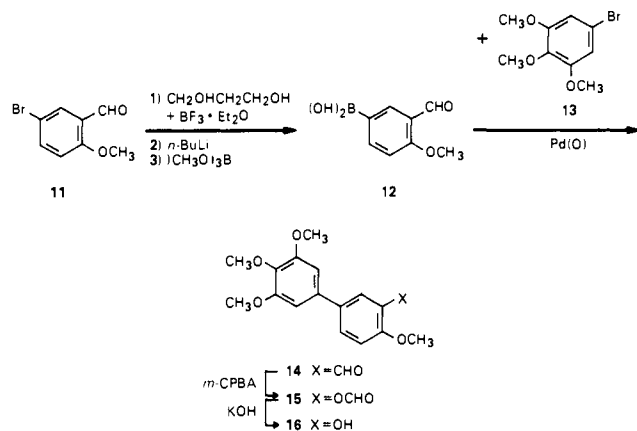
<sup>‡</sup> National Institutes of Health.

<sup>§</sup> United States Department of Agriculture.

(1) For a review, see: Hamel, E. Interactions of tubulin with small ligands. In *Microtubule Proteins*; Avila, J., Ed.; CRC Press: Boca Raton, FL, 1990; pp 89-191.

(2) The conformation of colchicine presented here is that described: Yeh, H. J. C.; Chrzanowska, M.; Brossi, A. The importance of the phenyl-tropolone 'aS' configuration in colchicine's binding to tubulin. *FEBS Lett.* 1988, 229, 82-86.

## Scheme I

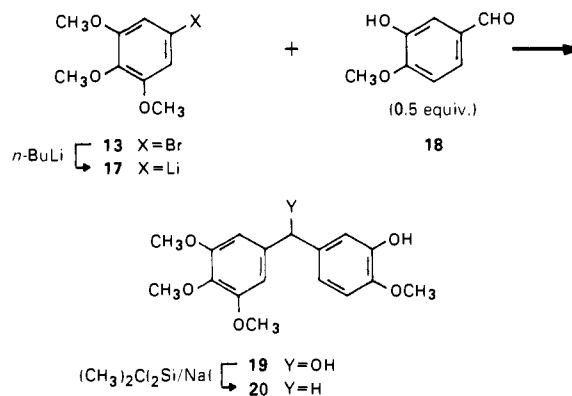


in this series of compounds. In 2 they are directly attached to each other. In 3-6 they are separated by one carbon atom. In 7 and 8 the bridge between the two phenyl rings consists of two carbon atoms. We therefore wondered whether there was an optimal separation of the two aromatic portions of these molecular structures, perhaps representing two domains which each bind to one of the two polypeptide chains of tubulin.

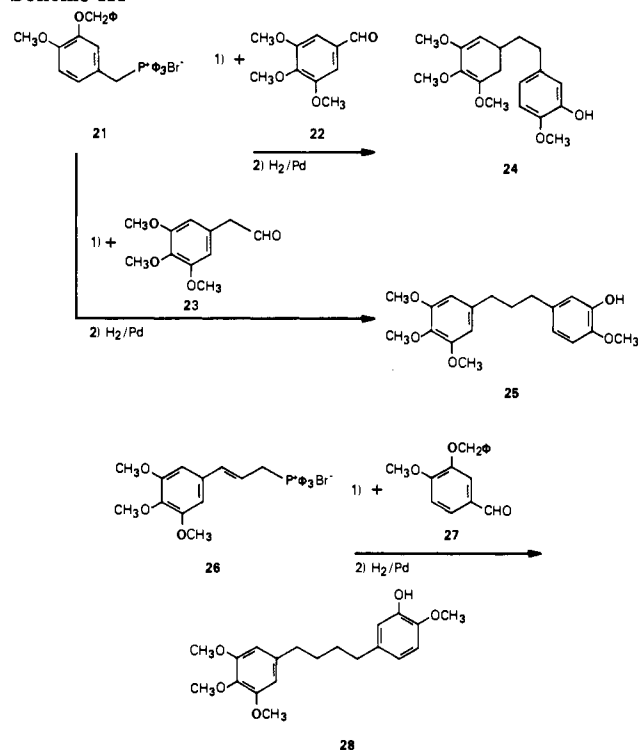
In this report we describe the synthesis of two series of compounds based on 3-6 and on 8 which differ only in the bridge separating the two ring systems. These compounds were then evaluated as potential antimetabolic agents on the basis of their abilities to inhibit *in vitro* tubulin polymerization and the binding of radiolabeled colchicine to tubulin. As standards in this evaluation we used 2-4 and 8, which is particularly potent as an inhibitor of the binding of radiolabeled colchicine to tubulin,<sup>10,11</sup> and thiocolchicine (10),<sup>14</sup> which has the greatest activity of all

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- Lin, C. M.; Singh, S. B.; Chu, P. S.; Dempey, R. O.; Schmidt, J. M.; Pettit, G. R.; Hamel, E. Interactions of tubulin with potent natural and synthetic analogs of the antimetabolic agent combretastatin: a structure-activity study. *Mol. Pharmacol.* 1988, 34, 200-208.
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## Scheme II



## Scheme III



the colchicine site agents as an inhibitor of tubulin polymerization.

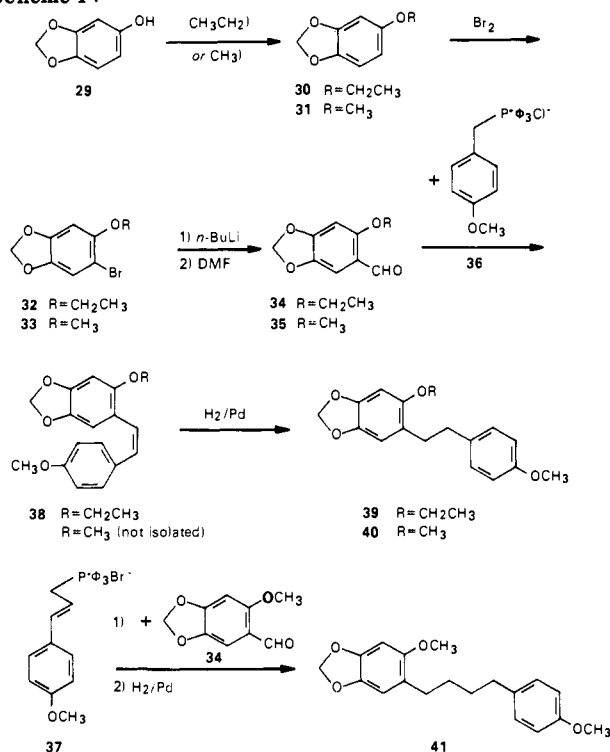
## Synthesis

Substituted diaryl compounds with bridge length varying from zero to four methylene groups were synthesized by the formation of carbon-carbon bonds between appropriately substituted aryl moieties.

**Combretastatin Series. Scheme I.** Protection of 5-bromo-*o*-anisaldehyde (11) as the acetal by treatment with 1,3-propanediol and  $\text{BF}_3 \cdot \text{Et}_2\text{O}$ , followed by treatment with *n*-BuLi and quenching with trimethyl borate yielded the aryl boronic acid 12 after acid workup. This was coupled with the aryl bromide 13 under Pd(O) catalysis to yield the anisaldehyde 14 by the Suzuki reaction.<sup>15</sup> Compound 14 was converted to the formate 15 with *m*-

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## Scheme IV



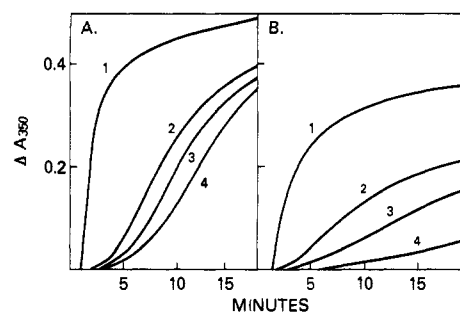
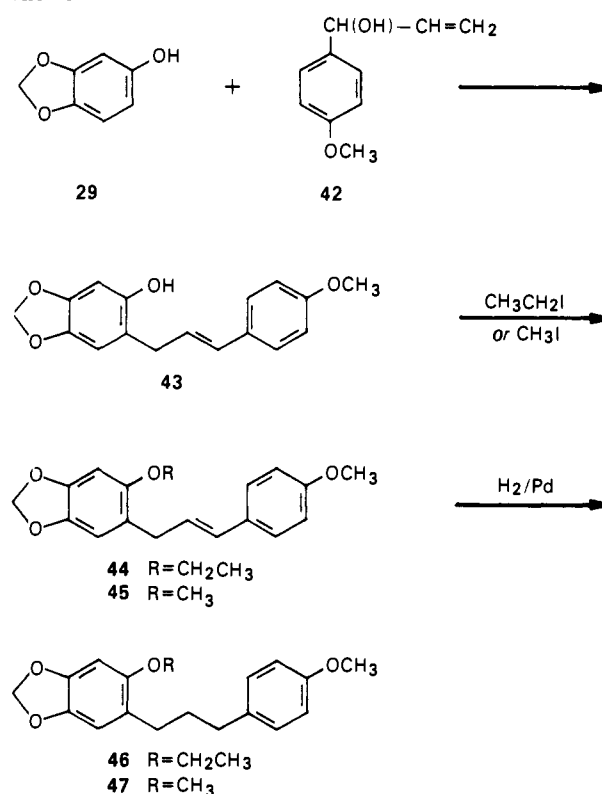
chloroperbenzoic acid. KOH was used to hydrolyze compound 15 to the desired phenol 16.

**Scheme II.** The aryllithium 17 was generated from the bromide 13 with *n*-BuLi. Compound 17 was coupled by nucleophilic addition to the substituted benzaldehyde 18 to yield the diaryl methanol 19. Reduction of 19 to the desired diaryl methane 20 was effected by reaction with dichloromethylsilane/sodium iodide as described by Wiggins.<sup>16</sup>

**Scheme III.** Wittig reaction of the phosphonium bromide 21<sup>17</sup> with aldehyde 22 or 23 yielded olefins which were hydrogenated without isolation under Pd/C catalysis to produce the desired compounds 24 and 25. A similar Wittig reaction with the phosphonium bromide 26<sup>18</sup> with aldehyde 27 (obtained by benzylation of 3-hydroxy-4-methoxybenzaldehyde) followed by hydrogenation over Pd afforded the desired compound 28. The syntheses of compounds 24, 25, and 28 have been outlined previously.<sup>10</sup>

**Benzodioxole Series. Scheme IV.** Sesamol (29) was reacted with ethyl or methyl iodide to yield the alkoxy derivatives 30 and 31. These were treated with Br<sub>2</sub> in CCl<sub>4</sub> to yield compounds 32 and 33, which were sequentially reacted with *n*-BuLi and DMF. This yielded the 5-alkoxy-piperonals 34 and 35. The Wittig reaction of triphenylphosphonium compounds 36<sup>19</sup> and 37<sup>18</sup> with compounds 34 and 35 yielded olefins, but only 38 was isolated. These were hydrogenated over Pd to yield the desired 39–41.

## Scheme V

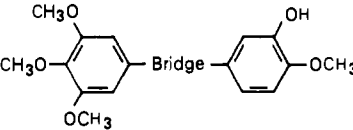


**Figure 1.** Comparison of the effects of compound 39 on tubulin polymerization under two similar reaction conditions. Experimental details are presented in text. Part A shows the results of the 37 °C/1.0 mM MgCl<sub>2</sub> reaction condition. Concentrations of compound 39 were as follows: curve 1, none; curve 2, 10 μM; curve 3, 40 μM; curve 4, 100 μM. Part B shows the results of the 30 °C/0.25 mM MgCl<sub>2</sub> reaction condition. Concentrations of compound 39 were as follows: curve 1, none; curve 2, 3 μM; curve 3, 4 μM; curve 4, 5 μM.

**Scheme V.** Aqueous acid catalyzed condensation of sesamol (29) with an appropriate benzylic alcohol,<sup>20,21</sup> followed by alkylation and hydrogenation, yielded the desired products. Sesamol (29) was condensed with 1-(4-methoxyphenyl)-2-propen-1-ol (42<sup>22</sup>) to yield the phenol 43. This was alkylated with ethyl or methyl iodide to yield, respectively, 44 and 45, which were hydrogenated over Pd

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**Table I.** Inhibitory Effects of Combretastatin Analogues on Tubulin Polymerization and on the Binding of Radiolabeled Colchicine to Tubulin


compd	bridge	inhibition of tubulin polymerization: IC <sub>50</sub> , μM (±SD) <sup>a</sup>		inhibition of colchicine binding: % inhibition (±SD) <sup>a</sup>	
		30 °C <sup>b</sup>	37 °C <sup>c</sup>	30 °C <sup>b</sup>	37 °C <sup>c</sup>
16	none	>40	>40	2.6 (±3)	0.3 (±0.5)
20	CH <sub>2</sub>	2.3 (±0.07)	7.7 (±1)	57 (±3)	38 (±6)
24	(CH <sub>2</sub> ) <sub>2</sub>	1.6 (±0.06)	3.5 (±0.4)	80 (±3)	67 (±4)
25	(CH <sub>2</sub> ) <sub>3</sub>	3.0 (±0.2)	11 (±0.6)	63 (±0.6)	41 (±4)
28	(CH <sub>2</sub> ) <sub>4</sub>	4.4 (±0.5)	30 (±2)	33 (±3)	18 (±6)
8	CH=CH (cis)	1.1 (±0.06)	2.5 (±0.09)	97 (±0.6)	96 (±0.6)
2	NM <sup>d</sup>	1.2 (±0.08)	2.6 (±0.3) <sup>e</sup>	68 (±1)	52 (±9)
10	NM <sup>d</sup>	0.64 (±0.2)	1.4 (±0.08)	52 (±5)	57 (±2)

<sup>a</sup>SD, standard deviation. <sup>b</sup>Reaction conditions were described in detail in the text. Reaction mixtures contained 0.25 mM MgCl<sub>2</sub>. Reaction temperature was 30 °C. <sup>c</sup>Reaction conditions were described in detail in the text. Reaction mixtures contained 1.0 mM MgCl<sub>2</sub>. Reaction temperature was 37 °C. <sup>d</sup>NM, not meaningful. <sup>e</sup>This value was obtained with a different tubulin preparation than that used in the rest of the experiments presented in the table.

to afford the desired 46 and 47.

### Biological Evaluation

**Effect of Reaction Temperature and Mg<sup>2+</sup> Concentration on Apparent Inhibitory Effects of Agents on Tubulin Polymerization.** We have been evaluating potential antitubulin agents by determining the concentration of a test compound required to inhibit the extent of glutamate-dependent tubulin polymerization by 50% (the IC<sub>50</sub> value). The reaction mixtures have contained 10 μM tubulin, and potent antimitotic compounds usually are active at concentrations substoichiometric to the tubulin concentration. Prior to the studies presented here, reaction mixtures contained 1.0 mM MgCl<sub>2</sub>, and the reaction temperature was 37 °C.<sup>10,14,23,24</sup> During the present work, however, we found that many of the compounds based on 3 and 4 could not be evaluated in this system. As shown in Figure 1A for compound 39, apparent inhibition of the control reaction responded minimally in a concentration-dependent manner, and we could find no concentration range in which the apparent turbidity plateau was reduced by over 50%. In addition, the polymerization product formed in the presence of drug was moderately more cold stable as compared with polymer formed in the absence of drug (data not presented). Since aberrant colchicine-dependent polymers have previously been described, particularly in reaction mixtures containing high Mg<sup>2+</sup> concentrations,<sup>25,26</sup> we examined the effects of both lower reaction temperature and lower Mg<sup>2+</sup> concentration on inhibition of polymerization by compound 39.

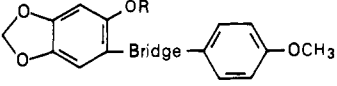
We found that a straightforward, concentration-dependent inhibition of polymerization occurred when the reaction temperature was lowered to 30 °C in the presence of 0.25 mM MgCl<sub>2</sub>, as shown in Figure 1B. This reaction condition was therefore used without difficulty with all the compounds described here.

The compounds with two phenyl rings, inspired by the combretastatins 7 and 8, all displayed progressive inhibitory patterns as a function of concentration in both the 37 °C/1.0 mM Mg<sup>2+</sup> system and the 30 °C/0.25 mM Mg<sup>2+</sup> system. IC<sub>50</sub> values for these agents were therefore obtained under both reaction conditions. In addition, all compounds were examined for inhibitory effects on the binding of radiolabeled colchicine to tubulin under both reaction conditions.

One major difference between the polymerization and colchicine-binding experiments should be noted. Because some compounds which interact with tubulin (notably most colchicinoids) bind relatively slowly to the protein, in polymerization assays we routinely preincubate tubulin with drugs prior to addition of GTP (required for polymerization) to minimize the possibility of not identifying a potent inhibitor which binds slowly. In the colchicine binding assays the radiolabeled ligand and potential inhibitor are mixed, then tubulin is added at 0 °C before the incubation is begun. Because colchicine both binds to and dissociates from tubulin relatively slowly, extent of inhibition of binding of the radiolabeled colchicine is a complex function of relative binding rates and relative affinities of the colchicine and the potential inhibitor.

**Structure-Activity Studies.** Table I summarizes the results obtained with the series of combretastatin analogues, with comparison to data obtained with compound 2, combretastatin A-4 (8), and thicolchicine (10). All experiments summarized in Table I (as well as Table II; see below) were obtained with the same tubulin preparation and were performed about the same time, except for the 37 °C polymerization study with compound 2. With the exception of the biphenyl compound 16, all the synthetic combretastatin analogues had activity as inhibitors both of tubulin polymerization and of colchicine binding. While none was as active as 2, combretastatin A-4 (8), or thicolchicine (10) as an inhibitor of polymerization, compounds 20, 24, and 25 were reasonably potent as inhibitors of colchicine binding.

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**Table II.** Inhibitory Effects of Benzodioxole Analogues on Tubulin Polymerization and on the Binding of Radiolabeled Colchicine to Tubulin


compd	bridge	inhibition of tubulin polymerization: IC <sub>50</sub> , μM (±SD) <sup>a</sup> at 30 °C <sup>b</sup>	inhibition of colchicine binding: % inhibition (±SD) <sup>a</sup>	
			30 °C <sup>b</sup>	37 °C <sup>c</sup>
R = CH <sub>2</sub> CH <sub>3</sub>				
3	CH(CH <sub>3</sub> )	1.8 (±0.09)	81 (±4)	72 (±3)
5	CH <sub>2</sub>	2.0 (±0.1)	67 (±5)	56 (±7)
39	(CH <sub>2</sub> ) <sub>2</sub>	3.8 (±0.4)	36 (±2)	26 (±6)
46	(CH <sub>2</sub> ) <sub>3</sub>	2.7 (±0.3)	40 (±5)	23 (±6)
38	CH=CH (cis)	>40	18 (±12)	8.0 (±7)
44	CH <sub>2</sub> CH=CH (trans)	1.9 (±0.1)	55 (±5)	38 (±7)
R = CH <sub>3</sub>				
4	CH(CH <sub>3</sub> )	2.5 (±0.3)	78 (±0.6)	62 (±3)
6	CH <sub>2</sub>	5.4 (±0.7)	42 (±4)	27 (±6)
40	(CH <sub>2</sub> ) <sub>2</sub>	8.8 (±2)	26 (±8)	16 (±5)
47	(CH <sub>2</sub> ) <sub>3</sub>	3.8 (±0.2)	40 (±6)	27 (±5)
41	(CH <sub>2</sub> ) <sub>4</sub>	7.5 (±2)	20 (±8)	11 (±2)
45	CH <sub>2</sub> CH=CH (trans)	3.9 (±0.5)	37 (±4)	27 (±3)

<sup>a</sup>SD, standard deviation. <sup>b</sup>Reaction conditions were described in detail in the text. Reaction mixtures contained 0.25 mM MgCl<sub>2</sub>. Reaction temperature was 30 °C. <sup>c</sup>Reaction conditions were described in detail in the text. Reaction mixtures contained 1.0 mM MgCl<sub>2</sub>. Reaction temperature was 37 °C.

There was a clear structure-activity correlation of bridge length with potency in this series of analogues, which was consistent in both assays and under both reaction conditions. Maximum activity was obtained with the two-carbon bridge analogue (24), while the one-carbon (20) and three-carbon (25) bridge analogues were somewhat less potent and nearly equivalent to each other (the one-carbon bridge compound was more effective than the three-carbon bridge analogue as an inhibitor of polymerization, while the latter appeared to be slightly more potent as an inhibitor of colchicine binding). Except for the inert biphenyl 16, the four-carbon bridge analogue (28) was the least effective inhibitor in this series of compounds.

There was a notable difference between the results obtained under the two reaction conditions. In the polymerization assay significantly lower IC<sub>50</sub> values were obtained with all effective agents at 30 °C with 0.25 mM Mg<sup>2+</sup> than at 37 °C with 1 mM Mg<sup>2+</sup>. At present it is not known whether this was caused predominantly by the lower temperature or the lower cation concentration. This reduction in the IC<sub>50</sub> value became progressively more marked as the IC<sub>50</sub> obtained at 37 °C with 1.0 mM Mg<sup>2+</sup> increased. Thus, in changing the reaction condition to 30 °C/0.25 mM Mg<sup>2+</sup> the IC<sub>50</sub> value of the highly potent agent thiocolchicine (10) was reduced about 2-fold (1.4 to 0.64 μM), that of compound 20 about 3-fold (7.7 to 2.3 μM), and that of compound 28 almost 7-fold (from 30 to 4.4 μM). Expressed another way, at 37 °C with 1.0 mM Mg<sup>2+</sup> thiocolchicine (10) was over 21 times as inhibitory as compound 28, while at 30 °C with 0.25 mM Mg<sup>2+</sup> thiocolchicine (10) was less than 7 times as inhibitory as 28. While less striking, all agents except thiocolchicine (10) also more potently inhibited colchicine binding at 30 °C with 0.25 mM Mg<sup>2+</sup> than at 37 °C with 1.0 mM Mg<sup>2+</sup>.

Table II summarizes the data obtained for the benzodioxole analogues, with two series of compounds (either an ethoxy or a methoxy substituent at position 2). As discussed above, these compounds could only be compared as inhibitors of polymerization in the 30 °C/0.25 mM Mg<sup>2+</sup>

condition; but their effects on radiolabeled colchicine binding were examined under both reaction conditions. On the basis of the results obtained with the combretastatin series, we anticipated maximum inhibition would occur with two-carbon bridge analogues. The opposite was observed, however: in both series, the two-carbon bridge analogues, 39 and 40, were the least potent inhibitors of tubulin polymerization (excluding the *cis*-ethene compound 38 in the ethoxy series).

In the two series of benzodioxole compounds, the same bridge modification had a qualitatively similar relative effect on activity as polymerization inhibitors. Without exception the 2-ethoxy compounds were more potent than the 2-methoxy compounds as inhibitors of tubulin polymerization: the ratio of IC<sub>50</sub> values of the former as compared with the latter ranged from 0.4 to 0.7 when compounds with the same bridge were compared with each other. In both series maximum inhibitory activity was observed with the original agents in the series, compounds 3 and 4, which have a CH(CH<sub>3</sub>) bridge. Less inhibitory activity was observed with a simple methylene bridge (compounds 5 and 6), and there was a further drop in activity with a two-carbon bridge (compounds 39 and 40). In the 2-ethoxy series, the analogue with an ethene bridge with the two rings in *cis* configuration relative to each other (compound 38) was noninhibitory. In contrast, in moving to a three-carbon bridge in both series there was a significant increase in inhibitory activity, whether the bridge was saturated (compounds 46 and 47) or contained a single double bond with the rings in *trans* configuration relative to each other (compounds 44 and 45). In the 2-ethoxy series the saturated three-carbon bridge analog 46 was less active than the unsaturated 44. The former was intermediate in activity between the one-carbon bridge analogue 5 and the two-carbon bridge analogue 39; and 44 was equivalent in activity to compound 5. In the 2-methoxy series compounds 45 and 47 had equivalent activity and were more inhibitory than the one-carbon bridge analogue 6. In the 2-methoxy series a four-carbon bridge analogue was prepared (compound 41). This was half as inhibitory as the three-carbon bridge analogue 45 and 47, but it was slightly more inhibitory than the two-carbon bridge analogue 40. Relative inhibitory effects of these compounds on the binding of radiolabeled colchicine to tubulin were generally in agreement with their inhibitory effects on the polymerization reaction.

In short, unlike with the combretastatin series, with the two benzodioxole series (which were consistent with each other) no obvious structure-activity pattern was obvious with respect to bridge separation of the two aryl ring components. In particular, the optimum two-carbon separation of the two phenyl rings in the combretastatin series was not reiterated in the benzodioxole series. In the latter, the analogues with a two-carbon bridge were the least potent agents in the series, with nearly equivalent activity obtained with one-carbon and three-carbon bridge compounds.

**Preliminary Cytotoxicity Evaluation.** All new compounds were compared for effects on the growth of L1210 murine leukemia cells, with measurement of increase in cell number after a 24-h incubation. Submicromolar IC<sub>50</sub> values were obtained in two independent experiments only with compounds 20, 24, and 25. The average values obtained with the three agents (less than 10% difference between the two experiments) were 0.2, 0.07, and 0.4 μM, respectively, and they all caused a significant rise in the number of cells arrested in mitosis at cytotoxic concentrations. In a single experiment with HL-60 human leu-

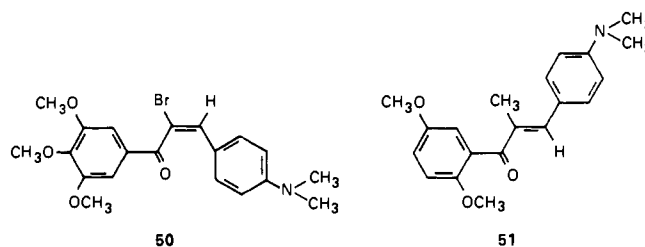
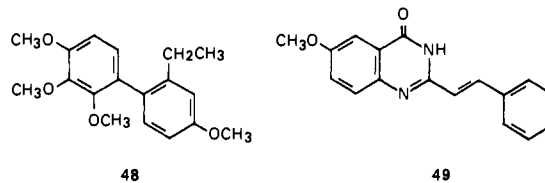
kemia cells, nearly identical  $IC_{50}$  values were obtained. These were 0.1, 0.05, and 0.3  $\mu M$ , respectively, for compounds 20, 24, and 25.

## Discussion

**Structure-Activity Correlates.** The purpose of this study was to determine whether the variable separation between two aryl ring systems in tubulin inhibitors such as occurs in compounds 2 (direct attachment), 3 and 4 (substituted methylene bridge), and 8 (2-carbon bridge) could be understood in terms of an optimal distance between the two rings. Our strategy was to prepare analogues of 8 on the one hand and of 3 and 4 on the other which differed only in the number of methylene units separating the two ring systems. Our results failed to yield such a simple explanation for the comparable inhibitory effects on tubulin polymerization observed with compounds 2, 4, and 8. While analogues of 8 were optimally active when the separation between the two phenyl rings was a two-carbon bridge, the two-carbon bridge analogues of compounds 3 and 4 were the least active inhibitors in their respective series. Nor did we succeed in preparing an analogue in any series more potent than the parent compounds.

Our data are also inconsistent with the hypothesis that direct attachment of the two rings always represents optimal separation, such as occurs in compound 2. Although we did not prepare an appropriate analogue of compounds 3 or 4 with direct ring attachment, we did prepare the biphenyl analogue of combretastatin, compound 16, which proved to be inert as a tubulin inhibitor. We should note, however, that significant inhibition of tubulin polymerization does occur with a similar biphenyl agent, compound 48, prepared by Boye et al.<sup>27</sup> We reexamined compound 48 in the current series of experiments, and an  $IC_{50}$  value of 7.5 ( $\pm$  0.6)  $\mu M$  was obtained in the 37 °C/1.0 mM  $MgCl_2$  system and of 2.3 ( $\pm$  0.4)  $\mu M$  in the 30 °C/0.25 mM  $MgCl_2$  system.

Besides the two series of compounds we have studied here, our results have important implications for other new colchicine site antimetabolic agents with similar diaryl structures. Two recent examples are derivatives of 2-styrylquinazolin-4(3*H*)-one,<sup>28,29</sup> such as compound 49, and chalcones,<sup>30,31</sup> such as compounds 50 and 51. Structure-activity evaluations of such series should ideally include different types and lengths of bridge as one factor to be



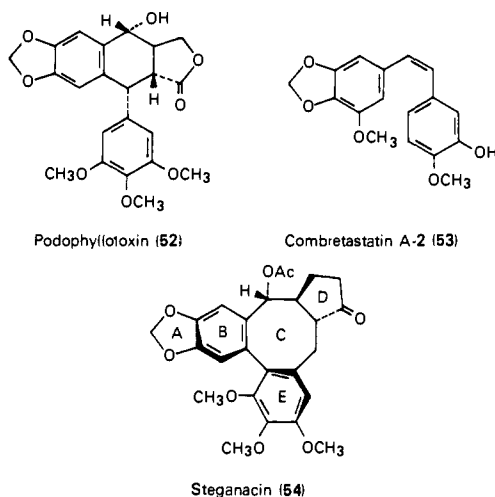
examined in the search for maximally active compounds. In the case of the styrylquinazolinone agents, a few bridge variants were available for study, and of these the most active agents had a two-carbon unsaturated bridge with the two aryl systems oriented *trans* to each other.<sup>28</sup> Reduction of the double bond caused a 3-fold loss of anti-tubulin activity, while an analogue with a one-carbon bridge was inactive. Neither a *cis* nor a three-carbon bridge analogue was prepared. The most active chalcones have a relatively complex three-carbon bridge, and active agents have been described with the aryl components oriented both *trans* and *cis* to each other. Their structural analogy to the combretastatins is obvious (especially considering that a dimethylamino group can replace the single methoxy group in the B ring of combretastatin A-4<sup>24</sup>), but effects of reducing the double bond, altering its position, or modifying bridge length have not been reported, nor were studies with an otherwise identical *cis*-*trans* pair. It should be noted that the *trans* isomer of combretastatin A-4 (8) was originally thought to have significant antitubulin activity,<sup>10</sup> but subsequent studies have shown this may have resulted from *trans* to *cis* isomerization.<sup>24</sup>

**Implications of Differential Inhibition of Polymerization under Suboptimal Reaction Conditions.** One possible explanation for the differences in the effect of ring separation distance on the apparent relative activities of different types of colchicine site drugs on tubulin polymerization is that more than two domains of the protein are involved in binding the aryl moieties. Such a model has been proposed to explain the failure of tropolonic compounds to affect the binding of podophyllotoxin (52) to tubulin, although they do feebly reduce the interaction of colchicine (1) with the protein.<sup>32,33</sup> We feel, however, that multiple domains represent an unlikely possibility, since such antimetabolic natural products as colchicine (1) versus cornigerine (9), combretastatin A-4 (8) versus combretastatin A-2 (53),<sup>11,34</sup> podophyllotoxin (52), and steganacin (54)<sup>35</sup> represent a virtual continuum of chemical structures

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which are unlikely to differ greatly in their specific binding site on tubulin.



A second possibility is that ring or bridge substituents have important roles to play in the interaction of the aryl moieties with tubulin, even to the point of altering the optimal separation between these moieties. Such substituents clearly have major impact on the interaction of colchicine-site agents with tubulin. In colchicine (1) itself alteration of A- or C-ring substituents yields compounds with both greater and lesser inhibitory effects than the parent compound;<sup>4,14,36,37</sup> and alteration of the B-ring substituents or conversion of the C ring from a tropolone to a phenyl ring can yield potent compounds which both bind to and dissociate from tubulin much more rapidly than colchicine.<sup>38-41</sup> In the combretastatin class of compounds substituent changes in the two phenyl rings have almost invariably resulted in agents with reduced activity, as has any modification in the basic two-carbon bridge (including all substituents so far examined).<sup>10,24</sup> Among benzodioxole compounds optimal C-ring substituent patterns vary with the precise structure of the remainder of the molecule.<sup>5,42</sup> It is also worth noting the contrast between the inactive compound 16 and the relatively good activity of compound 48.<sup>27</sup> This occurs with alteration in position of a single methoxy group, provided the hydroxyl and ethyl substituents are relatively unimportant. In the

case of combretastatin A-4 (8) elimination of the equivalent hydroxyl group causes only a minor loss of antitubulin activity.<sup>24</sup> With the biphenyls modeled on colchicine, such as compound 48, the *o*-ethyl group enhanced activity about 2-fold<sup>27</sup> while the *p*-methoxy group was required<sup>43</sup> for significant inhibitory effects on tubulin polymerization.

A third factor which should be considered is the possibility that differing apparent inhibitory effects on tubulin polymerization may reflect not only the relative binding affinity of agents for the colchicine site, but also represent somewhat differing effects of these compounds on the molecular mechanism of the polymerization reaction. Among postulated mechanisms for antimetabolic drugs are end poisoning of polymer by a tubulin-drug complex, copolymerization of drug-tubulin complex with unliganded tubulin resulting in distortion of the polymer structure, deficient nucleation, and accelerated disassembly reactions.<sup>1</sup> Even though different drugs bind at the same site on tubulin, they could have different mechanistic effects on polymerization. Possibly supporting such an explanation are the data showing somewhat different quantitative effects of the agents examined here as inhibitors of polymerization as compared with their inhibitory effects on the binding of radiolabeled colchicine to tubulin. Except for the problem of different association and dissociation rates of different agents as compared with colchicine as discussed above, this latter assay may more accurately reflect relative drug affinities for tubulin than does the polymerization assay if different mechanisms of assembly inhibition do exist.

A fourth factor which could partially explain the differing optimal separation of aryl moieties is suggested by the differing relative effects of compounds 2, 8, 10, 20, 24, 25, and 28 in the 30 °C/0.25 mM MgCl<sub>2</sub> system as compared with the 37 °C/1.0 mM MgCl<sub>2</sub> system (Table I). All compounds were more inhibitory in the former system as opposed to the latter, but the relative enhancement of activity was much more marked for the less active as opposed to the more active agents. Since both drug association and dissociation reaction rates should be reduced at the lower temperature, this finding suggests that a major effect of the modification of reaction conditions was to particularly reduce the dissociation rates of the less active compounds at 30 °C.

If, as we have suggested previously,<sup>11</sup> one aryl moiety binds on the  $\alpha$ -tubulin subunit and the other on the  $\beta$ -subunit, one could envisage the colchicine site as in a state of continuous flux, with the two subsites in continuous motion relative to each other. The binding rate and perhaps affinity of a specific agent for the colchicine site would depend on the length of time the protein remains in a favorable binding conformation. Once bound in the colchicine site, a ligand could perhaps restrict further mobility of the subunits relative to each other, with the dissociation rate reflecting the extent of this restriction. Thus at a lower temperature one would see a greater reduction in drug dissociation as compared with drug binding. This could explain both the generally enhanced inhibition of polymerization observed with all agents and the specifically greater enhancement observed with the weaker agents. With this model the poor dissociation of colchicine from tubulin would be explained as representing minimal mobility of the protein subunits in the tubulin-ligand complex. In effect, tubulin would be unable to expel colchicine from the binding site.

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Finally, we should note that a major goal in the evaluation of new agents in an *in vitro* assay, such as a tubulin polymerization assay, is the relatively inexpensive and accurate identification of compounds of potential medicinal value. The 37 °C/1.0 mM MgCl<sub>2</sub> system has been useful for this purpose, as most compounds which have substoichiometric IC<sub>50</sub> values (i.e., values below the tubulin concentration used) have significant cytotoxicity.<sup>10,14,23,24</sup> This does not appear to be true for the 30 °C/0.25 mM MgCl<sub>2</sub> polymerization system, which appears to be too sensitive. Most of the new agents yielded substoichiometric IC<sub>50</sub> values at 30 °C, including almost all the benzodioxole compounds. Yet only compounds 20, 24, and 25 possessed significant cytotoxicity. We therefore conclude that this modification of reaction conditions, required for the quantitative evaluation of the benzodioxole compounds with tubulin, is not satisfactory as a general screening procedure.

### Experimental Section

**Materials.** Combretastatin A-4 (8) and compound 2 were generously provided by Dr. G. R. Pettit of Arizona State University and Dr. T. J. Fitzgerald of Florida A & M University, respectively. Thiocolchicine (10) and [<sup>3</sup>H]colchicine were obtained from Rousell-Uclaf and Amersham, respectively. Compounds 3–6 were prepared as described previously.<sup>20,21</sup> Electrophoretically homogeneous bovine brain tubulin was purified as described elsewhere.<sup>44</sup> Magnesium-free glutamic acid was prepared as described previously,<sup>45</sup> and a stock 2 M solution at pH 6.6 was made by adding ultrapure NaOH, obtained from Alfa. A stock 2 M solution was also prepared from commercial monosodium glutamate, which was adjusted to pH 6.6 with HCl.

**Chemical Analysis.** Melting points were determined with a Thomas Hoover or Mel-temp II capillary melting point apparatus. IR spectra were obtained with a Perkin-Elmer 727B apparatus. <sup>1</sup>H NMR spectra were obtained with a Varian T-60 or Varian XL-200 (200 MHz) instrument. TLC was performed on silica gel GHLF plates obtained from Analtech. Column chromatography was performed on silica gel 60, 230–400 mesh, 60 Å, obtained from Merck. Microanalyses were performed by Galbraith Laboratories (Knoxville, TN) or Atlantic Microlab (Norcross, GA). We are indebted to Mr. W. L. White (Laboratory of Analytical Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases) for performing several of the CI mass spectral analyses.

**5-Boron *o*-Anisaldehyde (12).** A mixture of 5-bromo-*o*-anisaldehyde (11) (2.15 g, 10 mmol), 1,3-propanediol (3.6 mL, 50 mmol), and BF<sub>3</sub>·Et<sub>2</sub>O (0.3 mL) in toluene (5 mL) was heated (bath temp 140 °C) for 2 h. Reaction progress was monitored by TLC (SiO<sub>2</sub>, hexane/acetone 19:1). The reaction mixture was cooled, diluted with ether, and washed successively with saturated NaHCO<sub>3</sub> and water. Removal of the ether gave the protected benzaldehyde as a yellow, oily residue. This was dissolved in benzene, which was removed under reduced pressure: <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.72 (d, 1 H), 7.37 (dd, 1 H), 6.72 (d, 1 H), 5.81 (s, 1 H), 4.25 (m, 2 H), 3.98 (m, 2 H), 3.82 (s, 3 H), 2.23 (m, 1 H), 1.42 (m, 1 H). The protected benzaldehyde (2.53 g, 9.3 mmol) in THF (20 mL) under argon was cooled to -78 °C and treated with *n*-BuLi (1.6 M in hexanes, 10.2 mmol). Trimethyl borate (20 mmol) in THF was added and stirring continued at -70 °C. The reaction was monitored by TLC (SiO<sub>2</sub>, CHCl<sub>3</sub>/MeOH 9:1). After 2 h the reaction mixture was diluted with ether and water. The ether layer was washed twice with 10% HCl and then with water. Drying of the ether layer with Na<sub>2</sub>SO<sub>4</sub> and removal of the ether gave 12 as a white residue (1.25 g, 74% yield, mp 161–163 °C). The product was sparingly soluble in CHCl<sub>3</sub> and soluble in

methanol. It was recrystallized from methanol/CHCl<sub>3</sub>: <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 10.50 (s, 1 H), 8.24 (d, 1 H), 8.00 (d, 1 H), 7.05 (d, 1 H), 3.97 (s, 3 H).

**5-(3,4,5-Trimethoxyphenyl)-*o*-anisaldehyde (14).** Aqueous NaHCO<sub>3</sub> (5 mL of a 2 M solution) and 12 (0.9 g, 5 mmol) were added to a stirred solution of 3,4,5-trimethoxybromobenzene (13) (1.24 g, 5 mmol) and Pd(PPh<sub>3</sub>)<sub>4</sub> (0.17 g, 0.15 mmol) in toluene (10 mL) under argon. The mixture was stirred vigorously for 17 h at 70 °C. The reaction was monitored by TLC (SiO<sub>2</sub>, CHCl<sub>3</sub>). The reaction mixture was cooled and partitioned between CH<sub>2</sub>Cl<sub>2</sub> (50 mL) and aqueous Na<sub>2</sub>CO<sub>3</sub> (25 mL of a 2 M solution) containing concentrated ammonia (2.5 mL). The CH<sub>2</sub>Cl<sub>2</sub> layer was washed with saturated aqueous NaCl, the CH<sub>2</sub>Cl<sub>2</sub> solution was dried with Na<sub>2</sub>SO<sub>4</sub>, and removal of the solvent gave a residue which was purified by column chromatography. Compound 14 was obtained as an oil in a 60% yield (0.65 g): <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 10.52 (s, 1 H), 8.01 (d, 1 H), 7.74 (dd, 1 H), 7.05 (d, 1 H), 6.73 (s, 2 H), 3.97 (s, 3 H), 3.92 (s, 3 H), 3.88 (s, 3 H).

**2-Methoxy-5-(3,4,5-trimethoxyphenyl)phenol (16).** *m*-Chloroperbenzoic acid (85% pure) (0.29 g, 1.4 mmol) was added to a solution of 14 (1.14 mL, 0.344 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub>, and the mixture was heated at reflux overnight under nitrogen. The reaction was monitored by <sup>1</sup>H NMR [(CDCl<sub>3</sub>) δ 8.3 (s, 1 H, OCHO)]. The CH<sub>2</sub>Cl<sub>2</sub> was removed, and the residue was dissolved in EtOAc and washed successively with saturated aqueous NaHCO<sub>3</sub> and saturated NaCl solutions. The organic layer was separated and dried. Removal of the solvent afforded compound 15 as a yellowish oil. The formate 15 was hydrolyzed to the phenol 16 by dissolving in methanol and treating with 1.8 M KOH. Methanol was removed under reduced pressure. The resultant white solid was suspended in ether and acidified with 0.1 N HCl. The ether solution was separated and dried, and the solvent was removed affording 16 in 57% yield: <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.71 (d, 1 H), 7.49 (dd, 1 H), 6.95 (dd, 1 H), 6.74 (s, 2 H), 5.68 (d, 1 H), 4.91 (s, 6 H), 3.89 (s, 3 H), 3.87 (s, 3 H); MS *m/e* (M + 1) 291. Anal. (C<sub>16</sub>H<sub>18</sub>O<sub>5</sub>·0.6H<sub>2</sub>O) C, H.

**2-Methoxy-5-[(3,4,5-trimethoxyphenyl)methyl]phenol (20).** *n*-BuLi (1.6 M in hexanes, 1 mmol) was added to a solution of 3,4,5-trimethoxybromobenzene (13) (0.25 g, 1 mmol) in THF (2 mL) at -78 °C under argon. The mixture was stirred for 15 min, yielding 17, and 3-hydroxy-4-methoxy-benzaldehyde (18) (0.07 g, 0.45 mmol) in THF (2 mL) was added. Stirring continued at -78 °C for 1 h, and then overnight at room temperature. The reaction mixture was quenched with water acidified with 2 N HCl, and the reaction product was extracted successively into ether and into chloroform. The organic solutions were dried, and the solvents were removed. Purification by column chromatography (SiO<sub>2</sub>, CHCl<sub>3</sub>/MeOH 9:1) gave 55 mg (38% yield) of compound 19: <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 6.95–6.74 (m, 4 H), 6.60 (s, 1 H), 5.67 (s, 2 H), 4.54 (s, 1 H), 3.87–3.73 (m, 12 H).

(CH<sub>3</sub>)<sub>2</sub>Cl<sub>2</sub>Si (0.054 mL, 0.37 mmol) and compound 19 (55 mg, 0.17 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (0.85 mL) were added to a solution of CH<sub>2</sub>Cl<sub>2</sub>/(CH<sub>3</sub>)<sub>2</sub>O 1:1 (1.67 mL) containing NaI (0.11 g, 0.75 mmol) at 0 °C under argon. The mixture was stirred for 10 min, diluted with CH<sub>2</sub>Cl<sub>2</sub>, and washed successively with aqueous Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and water. The CH<sub>2</sub>Cl<sub>2</sub> solution was dried and the solvent removed, resulting in a residue which was purified by column chromatography (SiO<sub>2</sub>, CHCl<sub>3</sub>). Compound 20 was obtained in a 34% yield (17.5 mg): <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 6.82–6.63 (m, 3 H), 6.49 (s, 2 H), 5.60 (s, 1 H), 3.87–3.80 (m, 14 H); MS *m/e* (M + 1) 305. Anal. (C<sub>17</sub>H<sub>20</sub>O<sub>5</sub>) C, H.

**2-Methoxy-5-[2-(3,4,5-trimethoxyphenyl)ethyl]phenol (24).** Potassium *tert*-butoxide (0.25 g) was added in small portions to a stirred suspension of [3-(benzyloxy)-4-methoxybenzyl]triphenylphosphonium bromide (1.02 g, 1.8 mmol) (21) in anhydrous THF (10 mL) at room temperature. The mixture was stirred 10 min and cooled in an ice bath. A solution of 3,4,5-trimethoxybenzaldehyde (0.38 g, 1.9 mmol) (22) in THF (4 mL) was added dropwise to the reaction mixture. After 30 min the solution was diluted with water and extracted twice with ether. The ether extracts were combined, washed successively with water and brine, and dried (MgSO<sub>4</sub>), and the solvent was removed. The residue was dissolved in methanol and hydrogenated in the presence of 10% Pd/C overnight at room temperature. The solution was filtered through Celite, washed with CH<sub>2</sub>Cl<sub>2</sub>, and concentrated, and compound 24 (0.41 g, 72% yield) was isolated by column

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chromatography (SiO<sub>2</sub>, petroleum ether/ethyl acetate 1:1): IR (CHCl<sub>3</sub>) 3550, 2950, 2850, 1230, and 1010 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 6.73–6.42 (m, 3 H), 6.13 (s, 2 H), 5.53 (s, 1 H), 3.73 (s, 12 H), 2.75 (s, 4 H); MS *m/e* (M + 1) 319. Anal. (C<sub>18</sub>H<sub>22</sub>O<sub>5</sub>) C, H.

**2-Methoxy-5-[3-(3,4,5-trimethoxyphenyl)propyl]phenol (25).** Oxidation of 2-(3,4,5-trimethoxyphenyl)ethanol with pyridinium chlorochromate in methylene chloride gave 3,4,5-trimethoxyphenylacetaldehyde (23). Reaction sequences as described for preparation of 24 afforded 25 from 21 and 23 (52% yield): IR (CHCl<sub>3</sub>) 3540, 2940, 2401, and 1000 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 6.78–6.53 (m, 3 H), 6.30 (s, 2 H), 5.53 (s, 1 H), 3.80 (s, 12 H), 2.83–2.33 (m, 4 H), 2.17–1.67 (m, 2 H); MS *m/e* (M + 1) 333. Anal. (C<sub>19</sub>H<sub>24</sub>O<sub>5</sub>) C, H.

**2-Methoxy-5-[4-(3,4,5-trimethoxyphenyl)butyl]phenol (28).** Similar reaction sequences as described for compound 24 gave compound 28 from 26 and 27: IR (CHCl<sub>3</sub>) 3550, 2950, 2850, 1220, and 1000 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 6.77–6.55 (m, 3 H), 6.30 (s, 2 H), 5.57 (s, 1 H), 3.80 (s, 12 H), 2.73–2.30 (m, 4 H), 1.80–1.47 (m, 4 H); MS *m/e* (M + 1) 347. Anal. (C<sub>20</sub>H<sub>26</sub>O<sub>5</sub>) C, H.

**6-Ethoxypiperonal (34).** KOH (2.8 g, 50 mmol) in water (10 mL) and ethyl iodide (7.8 g, 50 mmol) were added consecutively to a solution of sesamol (29) (5 g, 36.2 mmol) in methanol (60 mL) at room temperature. The mixture was stirred for 48 h. Compound 30 was isolated, following addition of water and extraction into CHCl<sub>3</sub> (52% yield, mp 35–37 °C): <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 6.68 (d, 1 H), 6.47 (d, 1 H), 6.31 (dd, 1 H), 5.90 (s, 2 H), 3.94 (q, 2 H), 1.38 (t, 3 H). Treatment of 30 (91.5 g, 55 mmol) in CCl<sub>4</sub> (120 mL) at 0 °C with Br<sub>2</sub> (1 M in CCl<sub>4</sub>, 53 mL) for 2 h gave compound 32 (mp 49–51 °C, 41% yield following purification by vacuum distillation): <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 6.99 (s, 1 H), 6.55 (s, 1 H), 5.93 (s, 2 H), 4.10–3.90 (q, 2 H), 1.50–1.35 (t, 3 H). Compound 32 (1 g, 4.1 mmol) was treated for 10 min at –78 °C with *n*-BuLi (1.6 M solution in hexanes, 4.1 mmol) in anhydrous ether (8 mL). DMF (0.7 mL, 9.0 mmol, freshly distilled after drying over BaO) was added, and the reaction mixture was warmed to room temperature for 30 min. An additional 20 mL of ether was added, and the ether phase was washed with 0.1 N HCl. Compound 34 (mp 89–91 °C) was isolated from the ether layer in 88% yield: <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 10.27 (s, 1 H), 7.25 (s, 1 H), 6.56 (s, 1 H), 6.00 (s, 2 H), 4.18–4.00 (q, 2 H), 1.50–1.18 (t, 3 H).

***cis*-1-[2-Ethoxy-4,5-(methylenedioxy)phenyl]-2-(4-methoxyphenyl)ethane (38).** *n*-BuLi (1.6 M in hexanes, 0.67 mmol) and 34 (0.13 g, 0.67 mmol) were added consecutively to a suspension of (4-methoxybenzyl)triphenylphosphonium chloride (36) (0.28 g, 0.67 mmol) in anhydrous THF (10 mL) at –78 °C. The reaction mixture was stirred at –78 °C for 30 min, warmed to room temperature, stirred for 30 min, poured into ice (30 g), and extracted into ether. Purification by column chromatography (SiO<sub>2</sub>, hexane/EtOAc 49:1) and recrystallization from methanol yielded 38 (mp 96–98 °C): <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.43 (d, 2 H), 7.06 (s, 1 H), 6.92–6.82 (m, 4 H), 6.52 (s, 1 H), 5.92 (s, 2 H), 4.05–3.92 (q, 2 H), 3.82 (s, 3 H), 1.47–1.40 (t, 3 H); MS *m/e* (M + 1) 299. Anal. (C<sub>18</sub>H<sub>18</sub>O<sub>4</sub>) C, H.

**1-[2-Ethoxy-4,5-(methylenedioxy)phenyl]-2-(4-methoxyphenyl)ethane (39).** Compound 38 was dissolved in anhydrous toluene, 10% Pd on activated carbon was added, and the mixture was stirred under H<sub>2</sub> (1 atm) at room temperature overnight. The catalyst was removed by filtration and was washed with CH<sub>2</sub>Cl<sub>2</sub>. The combined filtrates were concentrated, and compound 39 was isolated as an oil following column chromatography (SiO<sub>2</sub>, 20% CHCl<sub>3</sub> in petroleum ether): <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.1–6.5 (m, 6 H), 5.9 (s, 2 H), 3.95 (q, 2 H), 3.8 (s, 3 H), 2.77 (m, 4 H), 1.4 (t, 3 H); MS *m/e* (M + 1) 301. Anal. (C<sub>18</sub>H<sub>20</sub>O<sub>4</sub>) C, H.

**1-[2-Methoxy-4,5-(methylenedioxy)phenyl]-2-(4-methoxyphenyl)ethane (40).** Compound 40 and its precursor 5-methoxypiperonal (35) were prepared exactly as described above for compounds 34 and 39, without, however, isolating the corresponding olefin, and in comparable yields: IR (CHCl<sub>3</sub>) 2940, 2840, 1250, and 1040 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 6.98 (d, 2 H), 6.68 (d, 2 H), 6.48 (s, 1 H), 6.40 (s, 1 H), 5.77 (s, 2 H), 3.70 (s, 6 H), 2.73 (s, 4 H); MS *m/e* (M + 1) 287. Anal. (C<sub>17</sub>H<sub>18</sub>O<sub>4</sub>) C, H.

**1-[2-Methoxy-4,5-(methylenedioxy)phenyl]-4-(4-methoxyphenyl)butane (41).** Potassium *tert*-butoxide (0.30 g, 2.7 mmol) was added in small portions to a stirred suspension of compound 37 (0.98 g, 2.0 mmol) in anhydrous THF (10 mL) at room temperature. Stirring continued for 10 min. Benzaldehyde 35 (0.40

g, 2.2 mmol) in THF (2 mL) was added dropwise to the reaction mixture. After 30 min the mixture was diluted with water (100 mL) and extracted with ether (75 mL, ×2). The combined ether extracts were washed successively with water (100 mL) and brine (100 mL), dried (MgSO<sub>4</sub>), filtered, and concentrated in vacuo. The residue was dissolved in methanol (15 mL) and hydrogenated in the presence of 10% Pd/C (20 mg) overnight at room temperature. The solution was filtered through Celite, washed with CH<sub>2</sub>Cl<sub>2</sub>, and concentrated in vacuo. Flash chromatography on SiO<sub>2</sub> (petroleum ether/EtOAc 4:1) gave 0.45 g (yield 71%, mp 56–58 °C) of compound 41: IR (CHCl<sub>3</sub>) 2930, 2850, 1240, and 1035 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.00 (d, 2 H), 6.71 (d, 2 H), 6.53 (s, 1 H), 6.42 (s, 1 H), 5.78 (s, 2 H), 3.72 (s, 3 H), 3.68 (s, 3 H), 2.78–2.18 (m, 4 H), 1.92–1.32 (m, 4 H); MS *m/e* (M + 1) 314. Anal. (C<sub>18</sub>H<sub>22</sub>O<sub>4</sub>) C, H.

**5-[3-(4-Methoxyphenyl)-2-propenyl]-1,3-benzodioxol-6-ol (43).** 1-(4-Methoxyphenyl)-2-propen-1-ol (42) (80 g) was combined with sesamol (29) (65.3 g), citric acid (20 g), ascorbic acid (5 g), and water (1.5 L) and refluxed for 4 h. The gummy material which separated from the cooled mixture was collected and dissolved in warm, wet benzene (200 mL). Compound 43 (45.5 g) crystallized as the mixture cooled and was recrystallized from benzene as colorless needles (mp 122–123 °C): <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.24 (d, 2 H), 6.80 (d, 2 H), 6.62 (s, 1 H), 6.42 (s, 1 H), 5.95–6.37 (m, 2 H), 5.85 (s, 2 H), 4.74 (s, 1 H, OH), 3.76 (s, 3 H), 3.40 (d, 2 H); MS *m/e* M<sup>+</sup> 284.1052. Anal. (C<sub>17</sub>H<sub>16</sub>O<sub>4</sub>) C, H.

**5-Ethoxy-6-[3-(4-methoxyphenyl)-2-propenyl]-1,3-benzodioxole (44).** A solution of compound 43 (3 g), ethyl iodide (5 mL), and potassium carbonate (5 g) in acetone (15 mL) was refluxed for 8 h. The mixture was concentrated and diluted with water. An oily product solidified and was collected and recrystallized from acetone/methanol to yield 2.7 g of the ethyl derivative 44 as colorless needles (mp 60–61 °C): <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.26 (d, 2 H), 6.78 (d, 2 H), 6.67 (s, 1 H), 6.47 (s, 1 H), 6.20–6.39 (m, 2 H), 5.82 (s, 2 H), 3.92 (q, 2 H), 3.76 (s, 3 H), 3.40 (d, 2 H), 1.34 (t, 3 H); MS *m/e* M<sup>+</sup> 312.1353. Anal. (C<sub>19</sub>H<sub>20</sub>O<sub>4</sub>) C, H.

Catalytic hydrogenation of 44 in the presence of 10% palladium carbon in tetrahydrofuran gave the dihydro derivative 46. This was crystallized from methanol as colorless needles (mp 29–30 °C): <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.10 (d, 2 H), 6.79 (d, 2 H), 6.62 (s, 1 H), 6.48 (s, 1 H), 5.84 (s, 2 H), 3.92 (q, 2 H), 3.76 (s, 3 H), 2.42–2.68 (m, 4 H), 1.68–2.00 (m, 2 H), 1.33 (t, 3 H); MS *m/e* M<sup>+</sup> 314.1530. Anal. (C<sub>19</sub>H<sub>22</sub>O<sub>4</sub>) C, H.

**5-Methoxy-6-[3-(4-methoxyphenyl)-2-propenyl]-1,3-benzodioxole (45).** A solution of compound 43 (2.4 g), methyl iodide (5 mL), and potassium carbonate (5 g) in acetone (15 mL) was refluxed for 5 h. The solid product was crystallized from acetone/methanol to yield 2.1 g of the methyl derivative 45 as colorless, brittle prisms (mp 91–92 °C): <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.26 (d, 2 H), 6.77 (d, 2 H), 6.66 (s, 1 H), 6.50 (s, 1 H), 6.10–6.31 (m, 2 H), 5.83 (s, 2 H), 3.74 (s, 6 H), 3.38 (d, 2 H); MS *m/e* M<sup>+</sup> 298.1211. Anal. (C<sub>18</sub>H<sub>18</sub>O<sub>4</sub>) C, H.

Catalytic hydrogenation of 45 in the presence of 10% palladium carbon gave the dihydro derivative 47. This was crystallized from methanol as colorless needles (mp 50–51 °C): <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.08 (d, 2 H), 6.78 (d, 2 H), 6.63 (s, 1 H), 6.47 (s, 1 H), 5.83 (s, 2 H), 3.74 (s, 3 H), 3.70 (s, 3 H), 2.42–2.68 (m, 4 H), 1.68–1.98 (m, 2 H); MS *m/e* M<sup>+</sup> 300.1378. Anal. (C<sub>18</sub>H<sub>20</sub>O<sub>4</sub>) C, H.

**Tubulin Polymerization Assay.** Assays at 37 °C were performed as described previously.<sup>14</sup> Reaction mixtures contained in a 0.24-mL volume 0.25 mg of tubulin (10 μM), 1.0 M commercial glutamate, 1.0 mM MgCl<sub>2</sub>, 4% (v/v) dimethyl sulfoxide, and varying concentrations of test compounds (all concentrations refer, however, to the final reaction volume of 0.25 mL). Samples were incubated at 37 °C for 15 min and then chilled on ice. GTP, required for tubulin polymerization, was added to each sample in 10 μL to a final concentration of 0.4 mM. Samples were transferred to Gilford 250 and 2400S recording spectrophotometers (four instruments per experiment; two control reactions per experiment) equipped with electronic temperature controllers. Baselines were established with the cuvettes held at 0 °C, and the reaction was initiated by a 75-s jump to 37 °C. Polymerization was followed for 20 min at 37 °C. IC<sub>50</sub> values, defined as the inhibitor concentration required to suppress the extent of polymerization by 50% after 20 min, were determined graphically. A minimum of three independent IC<sub>50</sub> values were obtained for

each compound, except that inactive compounds were generally examined only twice. In assays performed at 30 °C, magnesium-free glutamate was used, the final MgCl<sub>2</sub> concentration was 0.25 mM, and both the preincubation without GTP and the incubation in the spectrophotometers was performed at 30 °C.

**Colchicine Binding Assay.** The DEAE-cellulose filter assay was used.<sup>46</sup> Each 0.1-mL reaction mixture contained 0.1 mg/mL

tubulin (1.0 μM), 5 μM [<sup>3</sup>H]colchicine, 5 μM inhibitor (if present), 5% (v/v) dimethyl sulfoxide, and either 1.0 M commercial monosodium glutamate and 1.0 mM MgCl<sub>2</sub> (for 37 °C experiments) or 1.0 M magnesium-free monosodium glutamate and 0.25 mM MgCl<sub>2</sub> (for 30 °C experiments). Incubation was for 10 min at both temperatures. In individual experiments, triplicate samples were obtained for all data points, and each potential inhibitor was evaluated in at least three independent experiments.

**Inhibition of Growth of L1210 Murine Leukemia Cells and Evaluation of Cultures for Increased Numbers of Cells Arrested in Mitosis.** These experiments were performed as described previously.<sup>23</sup>

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## Structure-Activity Relationships for Inhibition of Papain by Peptide Michael Acceptors

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Two series of peptidyl Michael acceptors, *N*-Ac-L-Phe-NHCH<sub>2</sub>CH=CH-E with different electron withdrawing groups (E = CO<sub>2</sub>CH<sub>3</sub>, 1a; SO<sub>2</sub>CH<sub>3</sub>, 1b; CO<sub>2</sub>H, 1c; CN, 1d; CONH<sub>2</sub>, 1e; and C<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>, 1f) and R-NHCH<sub>2</sub>CH=CHCOOCH<sub>3</sub> with different recognition and binding groups (R = *N*-Ac-D-Phe, 2a; *N*-Ac-L-Leu, 3a; *N*-Ac-L-Met, 4a; PhCH<sub>2</sub>CH<sub>2</sub>CO, 5a; PhCO, 6a), were synthesized and evaluated as inactivators against papain. It was found that the inhibition of papain by peptidyl Michael acceptors is a general phenomenon and that the intrinsic chemical reactivity of the E group in the Michael acceptors has a direct effect on the kinetics of the inactivation process as reflected in  $k_2/K_1$ . At pH 6.2, the reactivity of papain toward the Michael acceptors is about 283 000-fold higher than the reactivity of the model thiol 3-mercaptopyruvate. This large increase in reactivity is attributable to at least 2 factors; one is the low apparent pK<sub>a</sub> of Cys-25 of papain, and the other is the recruitment of catalytic power by specific enzyme-substrate interactions. The unexpectedly high reactivity of 1c (E = COOH) was rationalized by proposing a direct interaction of the acid group with His-159 in the active site of papain. The unexpected inactivity of 1f (E = C<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>) as a Michael acceptor and its very powerful competitive inhibition of papain were rationalized by molecular graphics which showed the nitrophenyl moiety rotated out of conjugation with the olefin and interacting instead with the hydrophobic S<sub>1</sub>' region of papain. A plot of log( $k_2/K_1$ ) for 1a-6a vs log( $k_{cat}/K_m$ ) for analogous R-Gly-*p*-NA substrates was linear ( $r = 0.98$ ) with slope of 0.83, suggesting that binding energy from specific enzyme-ligand interactions can be used to drive the self-inactivation reaction to almost the same extent as it is used to drive catalysis.

Proteinase enzymes regulate many physiological functions such as digestion of dietary protein, blood coagulation, activation of physiologically active peptides from their inactive forms, and others.<sup>1</sup> Under physiological conditions, the activity of proteinase enzymes is controlled by means of storage in proenzyme forms, through their sequestration in subcellular vesicles, and by the presence of endogenous protein inhibitors.<sup>2</sup> Imbalances in these control mechanisms can result in excessive proteolytic activity with consequent alteration in physiological processes leading to pathological states. Thus agents which can inhibit selectively the action of a given proteinase without causing toxicity could potentially be developed into useful drugs. For example, synthetic inhibitors of angiotensin converting enzyme are being used clinically for the treatment of hypertension.<sup>3</sup> In addition, synthetic low molecular weight inhibitors have been used as investigative tools to characterize the catalytic functional groups, recognition specificity, catalytic mechanism and transition-state structure for proteinase enzymes.

The cysteine proteinase family includes the plant thiol proteinases such as papain, ficin, and actinidin, the mammalian lysosomal thiol proteinases such as cathepsin B,

H, and L, the mammalian calcium-activated neutral proteinases (calpains I and II), and certain viral-induced cysteine proteinases. The cathepsins play an important role in intracellular degradation of proteins and possibly in the activation of some peptide hormones.<sup>4</sup> Enzymes similar to cathepsins B and L are released from tumors and may be involved in tumor metastasis.<sup>5-7</sup> Defects in the regulation of cysteine proteinase activity by their endogenous inhibitors (cystatins) have been reported in connection with several disease processes, including cancer,<sup>8</sup> amyloidosis,<sup>9</sup> and muscular dystrophy.<sup>10</sup> Release of

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