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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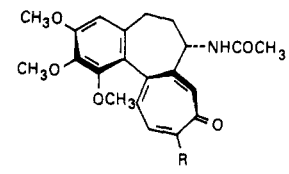
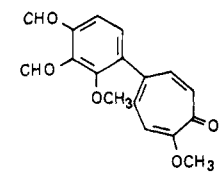
A number of cytostatic compounds (2–4, 7, and 8), which can be described as "diaryl", inhibit tubulin polymerization, cause cells to accumulate in mitotic arrest, and competitively inhibit the binding of colchicine to tubulin. They differ, however, in the separation of the two aryl moieties. To attempt 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 μM), 24 (0.07 μM), and 25 (0.4 μM). 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.[1] 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 1[2] whose tight binding to tubulin led to the original purification of the protein.[3] 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),[4] derivatives of benzyl-4,5-benzodioxole (such as 3–6),[5] combretastatin (7),[6-8] and, most potent of all, combretastatin A-4 (8).[9-11] 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)[12,13] are readily apparent in all cases.



R=OCH_3 Colchicine (1)
R=SCH_3 Thiocolchicine (10)



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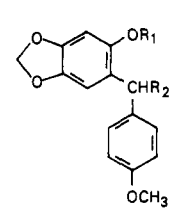
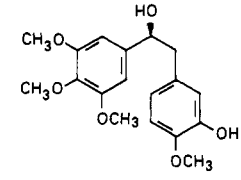
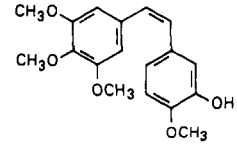


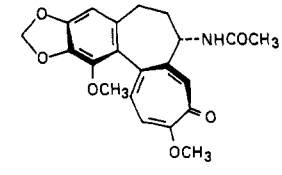
3 R_1=CH_2CH_3 R_2=CH_3
4 R_1=R_2=CH_3
5 R_1=CH_2CH_3 R_2=H
6 R_1=CH_3 R_2=H



Combretastatin (7)



Combretastatin A-4 (8)



Cornigerine (9)

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

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[‡] National Institutes of Health.
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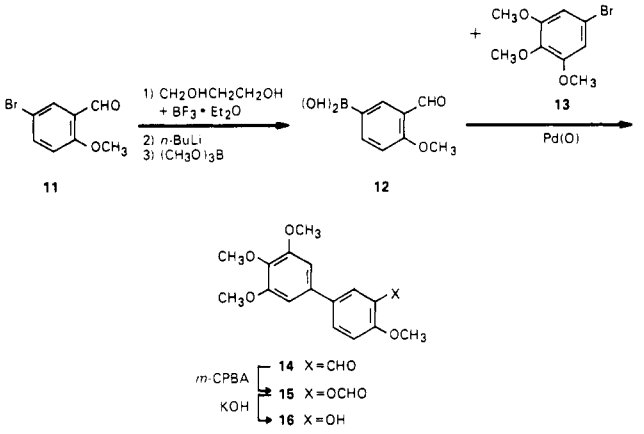
(1) For a review, see: Hamel, E. Interactions of tubulin with small ligands. In *Microtubule Proteins*; Avila, J., Ed.; CRC Press: Boca Raton, FL, 1990; pp 89–191.
(2) The conformation of colchicine presented here is that described: Yeh, H. J. C.; Chrzanowska, M.; Brossi, A. The importance of the phenyl-tropolone 'aS' configuration in colchicine's binding to tubulin. *FEBS Lett.* **1988**, *229*, 82–86.

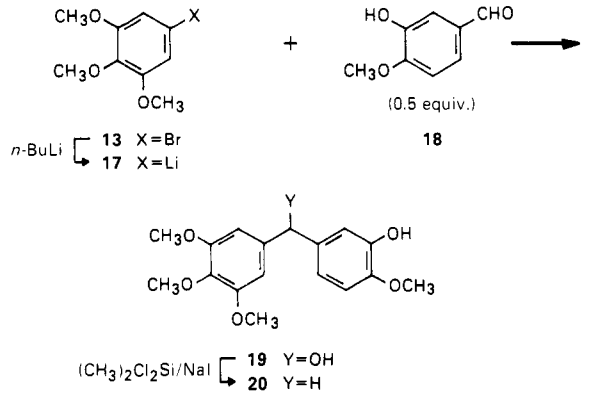
Scheme I



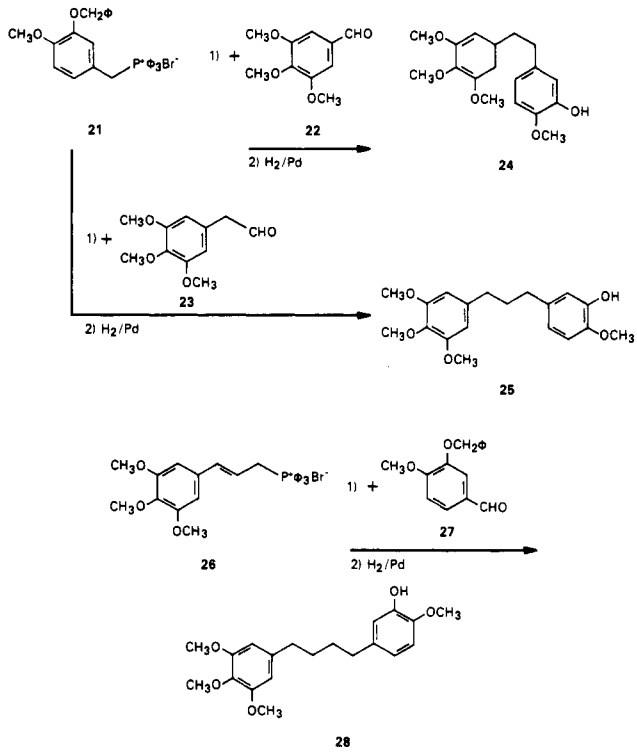
in this series of compounds. In **2** they are directly attached to each other. In **3–6** they are separated by one carbon atom. In **7** and **8** the bridge between the two phenyl rings consists of two carbon atoms. We therefore wondered whether there was an optimal separation of the two aromatic 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 antimitotic 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,[10,11] and thiocolchicine (**10**),[14] which has the greatest activity of all the colchicine site agents as an inhibitor of tubulin polymerization.

Scheme II



Scheme III



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 $BF_3 \cdot Et_2O$, 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.[15] Compound **14** was converted to the formate **15** with *m*-

(3) Weisenberg, R. C.; Borisy, G. G.; Taylor, E. W. The colchicine-binding protein of mammalian brain and its relation to microtubules. *Biochemistry* **1968**, *7*, 4466–4477.
(4) Fitzgerald, T. J. Molecular features of colchicine associated with antimitotic activity and inhibition of tubulin polymerization. *Biochem. Pharmacol.* **1976**, *25*, 1383–1387.
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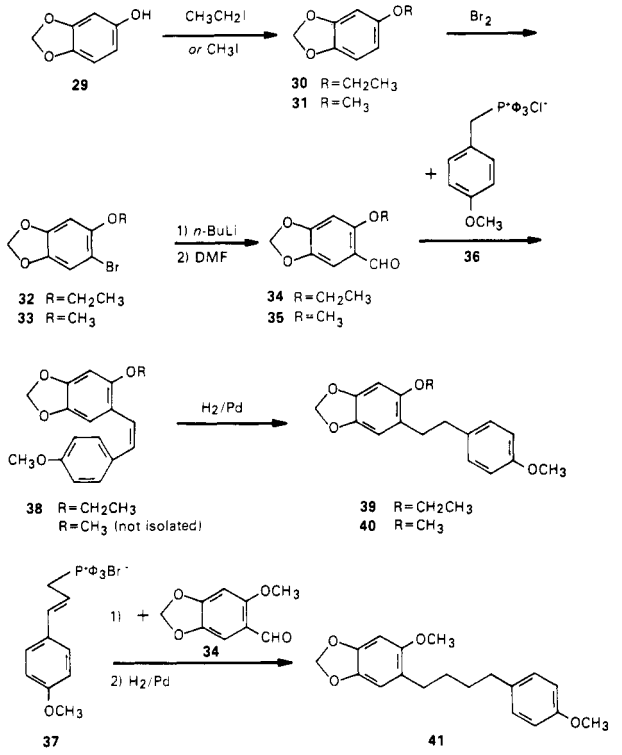
(6) Pettit, G. R.; Cragg, G. M.; Herald, D. L.; Schmidt, J. M.; Lohavanijaya, P. Isolation and structure of combretastatin. *Can. J. Chem.* **1982**, *60*, 1374–1376.
(7) Hamel, E.; Lin, C. M. Interactions of combretastatin, a new plant-derived antimitotic agent, with tubulin. *Biochem. Pharmacol.* **1983**, *32*, 3864–3867.
(8) Pettit, G. R.; Singh, S. B.; Cragg, G. M. Synthesis of natural (−)-combretastatin. *J. Org. Chem.* **1985**, *50*, 3404–3406.
(9) Pettit, G. R.; Singh, S. B.; Hamel, E.; Lin, C. M.; Alberts, D. S. Isolation and structure of the strong cell growth and tubulin inhibitor combretastatin A-4. *Experientia* **1989**, *45*, 209–211.
(10) Lin, C. M.; Singh, S. B.; Chu, P. S.; Dempcy, R. O.; Schmidt, J. M.; Pettit, G. R.; Hamel, E. Interactions of tubulin with potent natural and synthetic analogs of the antimitotic agent combretastatin: a structure-activity study. *Mol. Pharmacol.* **1988**, *34*, 200–208.
(11) Lin, C. M.; Ho, H. H.; Pettit, G. R.; Hamel, E. Antimitotic natural products combretastatin A-4 and combretastatin A-2: studies on the mechanism of their inhibition of the binding of colchicine to tubulin. *Biochemistry* **1989**, *28*, 6984–6991.
(12) Rosner, M.; Hsu, F.-L.; Brossi, A. Synthesis of two aromatic methylenedioxy-substituted colchicine congeners: elucidation of the structure of cornigerine as 2,3-(methylenedioxy)-2,3-didemethoxycolchicine. *J. Org. Chem.* **1981**, *46*, 3686–3688.
(13) Hamel, E.; Ho, H. H.; Kang, G.-J.; Lin, C. M. Cornigerine, a potent antimitotic *Colchicum* alkaloid of unusual structure. *Biochem. Pharmacol.* **1988**, *37*, 2445–2449.
(14) Muzaffar, A.; Brossi, A.; Lin, C. M.; Hamel, E. Antitubulin effects of derivatives of 3-demethylthiocolchicine, methylthio ethers of natural colchicinoids, and thioketones derived from thiocolchicine. Comparison with colchicinoids. *J. Med. Chem.* **1990**, *33*, 567–571.
(15) Miyaura, N.; Yanagi, T.; Suzuki, A. The palladium-catalyzed cross-coupling reaction of phenylboronic acid with haloarenes in the presence of bases. *Synth. Commun.* **1981**, *11*, 513–519.

Scheme IV



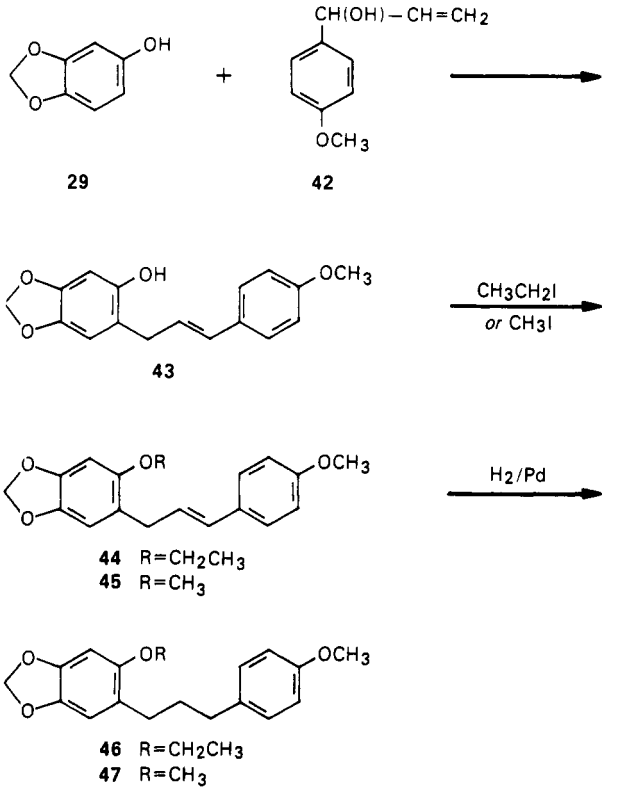
chloroperbenzoic acid. KOH was used to hydrolyze compound **15** to the desired phenol **16**.

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.[16]

Scheme III. Wittig reaction of the phosphonium bromide **21**[17] 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**[18] 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.[10]

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_2 in CCl_4 to yield compounds **32** and **33**, which were sequentially reacted with *n*-BuLi and DMF. This yielded the 5-alkoxypiperonals **34** and **35**. The Wittig reaction of triphenylphosphonium compounds **36**[19] and **37**[18] 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



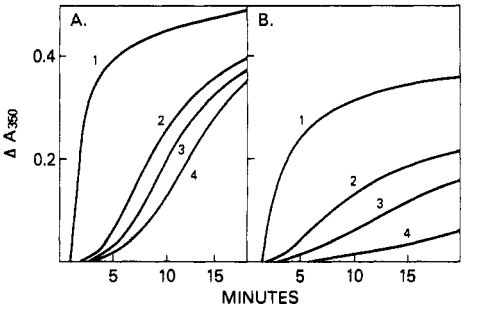


Figure 1. Comparison of the effects of compound **39** on tubulin polymerization under two similar reaction conditions. Experimental details are presented in text. Part A shows the results of the 37 °C/1.0 mM $MgCl_2$ reaction condition. Concentrations of compound **39** were as follows: curve 1, none; curve 2, 10 μM; curve 3, 40 μM; curve 4, 100 μM. Part B shows the results of the 30 °C/0.25 mM $MgCl_2$ 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,[20,21] followed by alkylation and hydrogenation, yielded the desired products. Sesamol (**29**) was condensed with 1-(4-methoxyphenyl)-2-propen-1-ol (**42**[22]) to yield the phenol **43**. This was alkylated with ethyl or methyl iodide to yield, respectively, **44** and **45**, which were hydrogenated over Pd

(16) Wiggins, J. M. A convenient procedure for the reduction of diarylmethanols with dichlorodimethylsilane/sodium iodide. *Synth. Commun.* **1988**, *18*, 741–749.
(17) Anapurna, G. S.; Deshpande, V. H. Synthesis of (±)-combretastatin. *Synth. Commun.* **1983**, *13*, 1075–1082.
(18) Eberbach, W.; Trostman, U. 6 and 8-π ring closure reactions of 2-oxaheptatrienyl dipoles: synthesis of 3-vinyl-2,3-dihydrofurans and 2,3(6,7)-dihydrooxepines. *Chem. Ber.* **1981**, *114*, 2979–3003.
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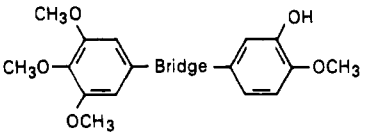
(20) Jurd, L.; Fye, R. L.; Morgan, J., Jr. New types of insect chemosterilants. Benzylphenols and benzyl-1,3-benzodioxole derivatives as additives to housefly diet. *J. Agric. Food Chem.* **1979**, *27*, 1007–1016.
(21) Jurd, L.; Narayanan, V. L.; Paull, K. D. In vivo antitumor activity of 6-benzyl-1,3-benzodioxole derivatives against the P388, L1210, B16, and M5076 murine models. *J. Med. Chem.* **1987**, *30*, 1752–1756.
(22) Jurd, L.; Roitman, J. N. Quinones and quinone methides — II: cyclization and hydration reactions of 2-cinnamyl-5-methoxy-1,4-benzoquinones. *Tetrahedron* **1978**, *34*, 57–62.

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_{50}, μM (±SD[a]) 30 °C[b]	37 °C[c]	inhibition of colchicine binding: % inhibition (±SD[a]) 30 °C[b]	37 °C[c]
16	none	>40	>40	2.6 (±3)	0.3 (±0.5)
20	CH_2	2.3 (±0.07)	7.7 (±1)	57 (±3)	38 (±6)
24	$(CH_2)_2$	1.6 (±0.06)	3.5 (±0.4)	80 (±3)	67 (±4)
25	$(CH_2)_3$	3.0 (±0.2)	11 (±0.6)	63 (±0.6)	41 (±4)
28	$(CH_2)_4$	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[d]	1.2 (±0.08)	2.6 (±0.3)[e]	68 (±1)	52 (±9)
10	NM[d]	0.64 (±0.2)	1.4 (±0.08)	52 (±5)	57 (±2)

[a] SD, standard deviation. [b] Reaction conditions were described in detail in the text. Reaction mixtures contained 0.25 mM $MgCl_2$. Reaction temperature was 30 °C. [c] Reaction conditions were described in detail in the text. Reaction mixtures contained 1.0 mM $MgCl_2$. Reaction temperature was 37 °C. [d] NM, not meaningful. [e] 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^{2+} 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_{50} 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 $MgCl_2$, and the reaction temperature was 37 °C.[10,14,23,24] 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^{2+} concentrations,[25,26] we examined the effects of both lower reaction temperature and lower Mg^{2+} 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 $MgCl_2$, as shown in Figure 1B. This reaction condition was therefore used without difficulty with all the compounds described here.

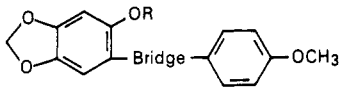
The compounds with two phenyl rings, inspired by the combretastatins **7** and **8**, all displayed progressive inhibitory patterns as a function of concentration in both the 37 °C/1.0 mM Mg^{2+} system and the 30 °C/0.25 mM Mg^{2+} system. IC_{50} values for these agents were therefore obtained under 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 thiocolchicine (**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 thiocolchicine (**10**) as an inhibitor of polymerization, compounds **20**, **24**, and **25** were reasonably potent as inhibitors of colchicine binding.

(23) Bai, R.; Pettit, G. R.; Hamel, E. Dolastatin 10, a powerful cytostatic peptide derived from a marine animal: inhibition of tubulin polymerization mediated through the vinca alkaloid binding domain. *Biochem. Pharmacol.* **1990**, *39*, 1941–1949.
(24) Cushman, M.; Nagarathnam, D.; Gopal, D.; Chakraborti, A. K.; Lin, C. M.; Hamel, E. Synthesis and evaluation of stilbene and dihydrostilbene derivatives as potential anticancer agents that inhibit tubulin polymerization. *J. Med. Chem.* **1991**, *34*, 2579–2588.
(25) Saltarelli, D.; Pantaloni, D. Polymerization of the tubulin-colchicine complex and guanosine 5′-triphosphate hydrolysis. *Biochemistry* **1982**, *21*, 2996–3006.
(26) Andreu, J. M.; Timasheff, S. N. Tubulin bound to colchicine forms polymers different from microtubules. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6753–6756.

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_{50}, μM (±SD[a]) at 30 °C[b]	inhibition of colchicine binding: % inhibition (±SD[a]) 30 °C[b]	37 °C[c]
		R = CH_2CH_3		
3	$CH(CH_3)$	1.8 (±0.09)	81 (±4)	72 (±3)
5	CH_2	2.0 (±0.1)	67 (±5)	56 (±7)
39	$(CH_2)_2$	3.8 (±0.4)	36 (±2)	26 (±6)
46	$(CH_2)_3$	2.7 (±0.3)	40 (±5)	23 (±6)
38	CH=CH (cis)	>40	18 (±12)	8.0 (±7)
44	CH_2CH=CH (trans)	1.9 (±0.1)	55 (±5)	38 (±7)
		R = CH_3		
4	$CH(CH_3)$	2.5 (±0.3)	78 (±0.6)	62 (±3)
6	CH_2	5.4 (±0.7)	42 (±4)	27 (±6)
40	$(CH_2)_2$	8.8 (±2)	26 (±8)	16 (±5)
47	$(CH_2)_3$	3.8 (±0.2)	40 (±6)	27 (±5)
41	$(CH_2)_4$	7.5 (±2)	20 (±8)	11 (±2)
45	CH_2CH=CH (trans)	3.9 (±0.5)	37 (±4)	27 (±3)

[a] SD, standard deviation. [b] Reaction conditions were described in detail in the text. Reaction mixtures contained 0.25 mM $MgCl_2$. Reaction temperature was 30 °C. [c] Reaction conditions were described in detail in the text. Reaction mixtures contained 1.0 mM $MgCl_2$. 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_{50} values were obtained with all effective agents at 30 °C with 0.25 mM Mg^{2+} than at 37 °C with 1 mM Mg^{2+}. 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_{50} value became progressively more marked as the IC_{50} obtained at 37 °C with 1.0 mM Mg^{2+} increased. Thus, in changing the reaction condition to 30 °C/0.25 mM Mg^{2+} the IC_{50} 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^{2+} thiocolchicine (**10**) was over 21 times as inhibitory as compound **28**, while at 30 °C with 0.25 mM Mg^{2+} 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^{2+} than at 37 °C with 1.0 mM Mg^{2+}.

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^{2+} 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_{50} 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 $CH(CH_3)$ 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 wih 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_{50} 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-

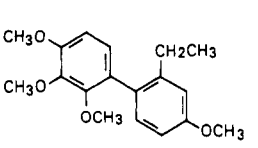
kemia cells, nearly identical IC_{50} values were obtained. These were 0.1, 0.05, and 0.3 μM, respectively, for compounds **20**, **24**, and **25**.

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.[27] We reexamined compound **48** in the current series of experiments, and an IC_{50} value of 7.5 (± 0.6) μM was obtained in the 37 °C/1.0 mM $MgCl_2$ system and of 2.3 (± 0.4) μM 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 antimitotic agents with similar diaryl structures. Two recent examples are derivatives of 2-styrylquinazolin-4(3*H*)-one,[28,29] such as compound **49**, and chalcones,[30,31] 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



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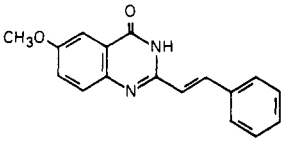

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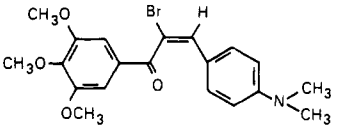

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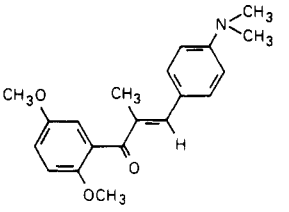

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examined in the search for maximally active compounds. In the case of the styrylquinazolinone agents, a few bridge variants were available for study, and of these the most active agents had a two-carbon unsaturated bridge with the two aryl systems oriented trans to each other.[28] Reduction of the double bond caused a 3-fold loss of antitubulin 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[24]), 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,[10] but subsequent studies have shown this may have resulted from trans to cis isomerization.[24]

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.[32,33] We feel, however, that multiple domains represent an unlikely possibility, since such antimitotic natural products as colchicine (**1**) versus cornigerine (**9**), combretastatin A-4 (**8**) versus combretastatin A-2 (**53**),[11,34] podophyllotoxin (**52**), and steganacin (**54**)[35] represent a virtual continuum of chemical structures

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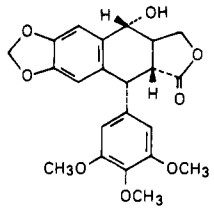
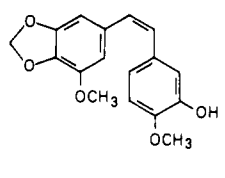
(35) The conformation of steganacin presented here is that described in Taafrout, M.; Rouessac, F.; Robin, J.-P. Araliangine, a new benzocyclooctadienolactonic lignan from *Steganotaenia araliacea*, Hochst. *Tetrahedron Lett.* **1983**, *24*, 197–200.

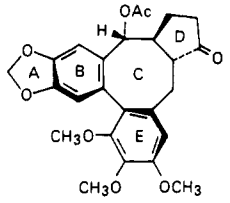
which are unlikely to differ greatly in their specific binding site on tubulin.



Podophyllotoxin (52)



Combretastatin A-2 (53)



Steganacin (54)

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;[4,14,36,37] 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.[38-41] 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).[10,24] Among benzodioxole compounds optimal C-ring substituent patterns vary with the precise structure of the remainder of the molecule.[5,42] It is also worth noting the contrast between the inactive compound **16** and the relatively good activity of compound **48**.[27] 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.[24] With the biphenyls modeled on colchicine, such as compound **48**, the *o*-ethyl group enhanced activity about 2-fold[27] while the *p*-methoxy group was required[43] 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 antimitotic 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.[1] 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 $MgCl_2$ system as compared with the 37 °C/1.0 mM $MgCl_2$ 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,[11] one aryl moiety binds on the α-tubulin subunit and the other on the β-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 $MgCl_2$ sytem has been useful for this purpose, as most compounds which have substoichiometric IC_{50} values (i.e., values below the tubulin concentration used) have significant cytotoxicity.[10,14,23,24] This does not appear to be true for the 30 °C/0.25 mM $MgCl_2$ polymerization system, which appears to be too sensitive. Most of the new agents yielded substoichiometric IC_{50} 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 [^{3}H]colchicine were obtained from Rousell-Uclaf and Amersham, respectively. Compounds **3–6** were prepared as described previously.[20,21] Electrophoretically homogeneous bovine brain tubulin was purified as described elsewhere.[44] Magnesium-free glutamic acid was prepared as described previously,[45] 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. ^{1}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 A, 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-Boronic *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_3·Et_2O (0.3 mL) in toluene (5 mL) was heated (bath temp 140 °C) for 2 h. Reaction progress was monitored by TLC (SiO_2, hexane/acetone 19:1). The reaction mixture was cooled, diluted with ether, and washed successively with saturated $NaHCO_3$ 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: ^{1}H NMR ($CDCl_3$) 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_2, $CHCl_3$/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_2SO_4 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 $CHCl_3$ and soluble in methanol. It was recrystallized from methanol/$CHCl_3$: ^{1}H NMR ($CDCl_3$) 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 $NaHCO_3$ (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 $Pd(PPh_3)_4$ (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_2, $CHCl_3$). The reaction mixture was cooled and partitioned between CH_2Cl_2 (50 mL) and aqueous Na_2CO_3 (25 mL of a 2 M solution) containing concentrated ammonia (2.5 mL). The CH_2Cl_2 layer was washed with saturated aqueous NaCl, the CH_2Cl_2 solution was dried with Na_2SO_4, 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): ^{1}H NMR ($CDCl_3$) 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_2Cl_2, and the mixture was heated at reflux overnight under nitrogen. The reaction was monitored by ^{1}H NMR [($CDCl_3$) δ 8.3 (s, 1 H, OCHO)]. The CH_2Cl_2 was removed, and the residue was dissolved in EtOAc and washed successively with saturated aqueous $NaHCO_3$ 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: ^{1}H NMR ($CDCl_3$) 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. ($C_{16}H_{18}O_5$·$0.6H_2O$) 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_2, $CHCl_3$/MeOH 9:1) gave 55 mg (38% yield) of compound **19**: ^{1}H NMR ($CDCl_3$) 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).

$(CH_3)_2Cl_2Si$ (0.054 mL, 0.37 mmol) and compound **19** (55 mg, 0.17 mmol) in CH_2Cl_2 (0.85 mL) were added to a solution of CH_2Cl_2/$(CH_3)_2O$ 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_2Cl_2, and washed successively with aqueous $Na_2S_2O_3$ and water. The CH_2Cl_2 solution was dried and the solvent removed, resulting in a residue which was purified by column chromatography (SiO_2, $CHCl_3$). Compound **20** was obtained in a 34% yield (17.5 mg): ^{1}H NMR ($CDCl_3$) 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. ($C_{17}H_{20}O_5$) 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 ($MgSO_4$), 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_2Cl_2, and concentrated, and compound **24** (0.41 g, 72% yield) was isolated by column

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chromatography (SiO_2, petroleum ether/ethyl acetate 1:1): IR ($CHCl_3$) 3550, 2950, 2850, 1230, and 1010 cm^{-1}; ^1H NMR ($CDCl_3$) 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. ($C_{18}H_{22}O_5$) 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 ($CHCl_3$) 3540, 2940, 2401, and 1000 cm^{-1}; ^1H NMR ($CDCl_3$) 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. ($C_{19}H_{24}O_5$) 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 ($CHCl_3$) 3550, 2950, 2850, 1220, and 1000 cm^{-1}; ^1H NMR ($CDCl_3$) 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. ($C_{20}H_{26}O_5$) 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 $CHCl_3$ (52% yield, mp 35–37 °C): ^1H NMR ($CDCl_3$) 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** (9.15 g, 55 mmol) in CCl_4 (120 mL) at 0 °C with Br_2 (1 M in CCl_4, 53 mL) for 2 h gave compound **32** (mp 49–51 °C, 41% yield following purification by vacuum distillation): ^1H NMR ($CDCl_3$) 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: ^1H NMR ($CDCl_3$) 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)ethene (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_2, hexane/EtOAc 49:1) and recrystallization from methanol yielded **38** (mp 96–98 °C): ^1H NMR ($CDCl_3$) 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. ($C_{18}H_{18}O_4$) 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_2 (1 atm) at room temperature overnight. The catalyst was removed by filtration and was washed with CH_2Cl_2. The combined filtrates were concentrated, and compound **39** was isolated as an oil following column chromatography (SiO_2, 20% $CHCl_3$ in petroleum ether): ^1H NMR ($CDCl_3$) 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. ($C_{18}H_{20}O_4$) 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 ($CHCl_3$) 2940, 2840, 1250, and 1040 cm^{-1}; ^1H NMR ($CDCl_3$) 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. ($C_{17}H_{18}O_4$) 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 ($MgSO_4$), 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_2Cl_2, and concentrated in vacuo. Flash chromatography on SiO_2 (petroleum ether/EtOAc 4:1) gave 0.45 g (yield 71%, mp 56–58 °C) of compound **41**: IR ($CHCl_3$) 2930, 2850, 1240, and 1035 cm^{-1}; ^1H NMR ($CDCl_3$) 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. ($C_{19}H_{22}O_4$) 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): ^1H NMR ($CDCl_3$) 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 $M^{\cdot+}$ 284.1052. Anal. ($C_{17}H_{16}O_4$) 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): ^1H NMR ($CDCl_3$) 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 $M^{\cdot+}$ 312.1353. Anal. ($C_{19}H_{20}O_4$) 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): ^1H NMR ($CDCl_3$) 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 $M^{\cdot+}$ 314.1530. Anal. ($C_{19}H_{22}O_4$) 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): ^1H NMR ($CDCl_3$) 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 $M^{\cdot+}$ 298.1211. Anal. ($C_{18}H_{18}O_4$) 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): ^1H NMR ($CDCl_3$) 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 $M^{\cdot+}$ 300.1378. Anal. ($C_{18}H_{20}O_4$) C, H.

Tubulin Polymerization Assay. Assays at 37 °C were performed as described previously.[14] Reaction mixtures contained in a 0.24-mL volume 0.25 mg of tubulin (10 μM), 1.0 M commercial glutamate, 1.0 mM $MgCl_2$, 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_{50} 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_{50} 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 $MgCl_2$ 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.[46] Each 0.1-mL reaction mixture contained 0.1 mg/mL tubulin (1.0 μM), 5 μM [^{3}H]colchicine, 5 μM inhibitor (if present), 5% (v/v) dimethyl sulfoxide, and either 1.0 M commercial monosodium glutamate and 1.0 mM $MgCl_2$ (for 37 °C experiments) or 1.0 M magnesium-free monosodium glutamate and 0.25 mM $MgCl_2$ (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.[23]

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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-$NHCH_2CH$=CH-E with different electron withdrawing groups (E = CO_2CH_3, **1a**; SO_2CH_3, **1b**; CO_2H, **1c**; CN, **1d**; $CONH_2$, **1e**; and C_6H_4-*p*-NO_2, **1f**) and R-$NHCH_2CH$=$CHCOOCH_3$ with different recognition and binding groups (R = *N*-Ac-D-Phe, **2a**; *N*-Ac-L-Leu, **3a**; *N*-Ac-L-Met, **4a**; $PhCH_2CH_2CO$, **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_i. 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-mercaptopropionate. This large increase in reactivity is attributable to at least 2 factors; one is the low apparent pK_a 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_6H_4-*p*-NO_2) 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_1' region of papain. A plot of log (k_2/K_i) 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.[1] 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.[2] 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.[3] 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.[4] Enzymes similar to cathepsins B and L are released from tumors and may be involved in tumor metastasis.[5–7] Defects in the regulation of cysteine proteinase activity by their endogenous inhibitors (cystatins) have been reported in connection with several disease processes, including cancer,[8] amyloidosis,[9] and muscular dystrophy.[10] Release of

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