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ISOLATION, STRUCTURE, AND SYNTHESIS OF COMBRETASTATINS A-1 AND B-1, POTENT NEW INHIBITORS OF MICROTUBULE ASSEMBLY, DERIVED FROM *COMBRETUM CAFFRUM*[1,2]

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ABSTRACT.—The principal antineoplastic constituent of the South African tree *Combretum caffrum* has been isolated and designated combretastatin A-1. The structure of this new *cis*-stilbene [**1a**] was unequivocally established by X-ray crystal structure determination and total synthesis. A Wittig reaction sequence [**5d** and **6**] in THF comprised the synthetic key step (92.5% yield) and provided a very favorable 9:1 ratio of the *cis:trans* [**1c:2c**, geometrical isomers]. Selective hydrogenation of combretastatin A-1 [**1a**] afforded combretastatin B-1 [**3**], a companion cell growth inhibitory constituent of *C. caffrum*. Combretastatin A-1 [**1a**] provided 26-29% life extension at 2.75-11 mg/kg dose levels with ED_{50} 0.99 μg/ml against the murine P-388 lymphocytic leukemia in vivo and in vitro systems. Both combretastatin A-1 and combretastatin B-1 are potent inhibitors of microtubule assembly in vitro and among the most potent inhibitors of the binding of colchicine to tubulin yet described. The structural simplicity and ready synthesis of combretastatin A-1 and combretastatin B-1 suggest that these new biosynthetic products will become useful in a variety of biological endeavors.

Tropical and subtropical shrubs and trees of the Combretaceae family represent a practically unexplored reservoir of new substances with potentially useful biological properties. Illustrative is the genus *Combretum* with 25 species (10% of the total) known in the primitive medical practices of Africa and India for uses as diverse as treating leprosy (1) (*Combretum* sp. roots) and cancer[6] (*Combretum latifolium*). But only a few species, principally *Combretum micranthum* (2,3) (used in northern Zimbabwe for mental illness) and *Combretum zeyheri* (for scorpion invenomation) (4), have received any scientific study. The present investigation was undertaken to uncover the murine P-388 lymphocytic leukemia (PS system) inhibitory constituents of *Combretum caffrum* (Eckl. and Zeyh) Kuntze (syn. *Combretum salicifolium* E. Mey), a potentially useful lead arising from the U.S National Cancer Institute's worldwide exploratory survey of plants. In South Africa this tree is known by the Zulu as "Mdubu" (used as a charm) and is otherwise known as bushveld willow, bushwillow, and "rooiblaar." The timber is principally used on African farms as scrap wood and fuel (1). Interestingly, honey arising from the nectar of this tree is strongly bitter, but no problems have been recorded from human consumption.

In 1981, we succeeded in isolating a new substance named combretastatin that caused pronounced astrocyte reversal in the NCI astrocytoma bioassay and was found to

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[1]Contribution 124 of the series Antineoplastic Agents. For part 123, refer to J.B. Smith, L. Smith, and G.R. Pettit, *Biochem. Biophys. Res. Commun.*, **132**, 939 (1985).

[2]This article commemorates the 50th year of publication of the *Journal of Natural Products* (formerly *Lloydia*).

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[6]Private communication from Drs. J.A. Duke and J.K. Win, USDA, Beltsville, Maryland.

be *R*(−)-1-(3,4,5-trimethoxyphenyl)-2-(3′-hydroxy,4′-methoxyphenyl) ethanol. Meanwhile, attention has been focused on uncovering the principal PS in vivo active constituent(s) of *C. caffrum* fractions where combretastatin was not responsible for the biological activity. The problem was complicated by eventual loss of in vivo activity in fractions obtained from the original large scale extraction of the stems and fruit parts that led to combretastatin (5,6). Consequently, the large scale (77 kg) extraction of *C. caffrum* was repeated using the stem-wood and CH_2Cl_2/MeOH as solvent (7).

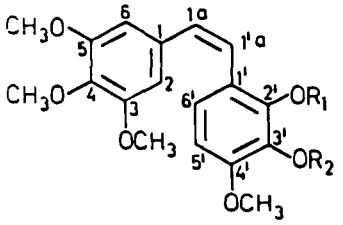
The CH_2Cl_2 fraction obtained by diluting the CH_2Cl_2/MeOH extract with H_2O was subjected to solvent partition between MeOH-H_2O (9:1↦3:2) with hexane/CH_2Cl_2. By this means the PS in vivo activity (38-41% life extension at 25-50 mg/kg and ED_{50} 0.21 μg/ml) was concentrated in the CH_2Cl_2 fraction. Steric exclusion chromatography of the active CH_2Cl_2 fraction in MeOH on Sephadex LH-20 led to a fraction (30.6 g) preserving the PS in vivo activity. At this stage it was found most effective to proceed by partition chromatography on Sephadex LH-20 with hexane-toluene-MeOH (3:1:1) as mobile phase. The PS activity (30-48% life extension at 12.5-50 mg/kg) was nicely concentrated in an active fraction that was further purified by Si gel column chromatography and elution with hexane-EtOAc (3:1). After recrystallization, the principal active component, herein named combretastatin A-1, was obtained in 9.1×10^{-4}% yield (0.70 g), and it was unequivocally assigned structure **1a** (NSC 600032, PS 26-29% increase in life extension at 2.75-11 mg/kg dose levels and ED_{50} 0.99 μg/ml, experiments at higher dose levels are now in progress); the companion cell growth inhibitory factor (PS ED_{50} 1.7 μg/ml), designated combretastatin B-1 (NSC 601291), was assigned structure **3**. The basis for these conclusions is as follows.

Both the uv and ir spectra of combretastatins A-1 and B-1 suggested aromatic systems, and this was further supported by the high resolution eims and molecular formula $C_{18}H_{20}O_6$ and $C_{18}H_{22}O_6$, respectively. The 400 MHz ^{1}H-nmr spectrum exhibited signals for four methoxyl group protons and, in general, indicated that combretastatin B-1 was a dihydro derivative of the A-1. Thus, further structural efforts were concentrated on determining the structure of combretastatin A-1.

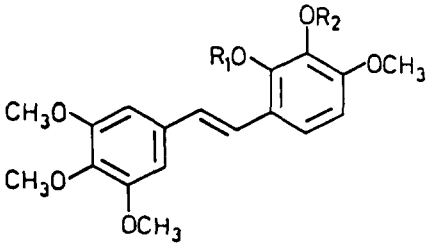
The 400 MHz ^{1}H-nmr spectrum of combretastatin A-1 exhibited two magnetically identical and relatively shielded aromatic protons at δ 6.460, two AB spin systems totalling four protons in aromatic region with one of these appearing as a doublet at δ 6.310 (J=8.64 Hz) and its counterpart at δ 6.691 typical of two *ortho* coupled aromatic protons. The other AB spin system showed doublets at δ 6.453 and 6.523 (J=12.2 Hz each). A two-proton signal at δ 5.438 was readily exchanged for deuterium upon adding D_2O suggesting the presence of phenolic groups, and that observation was confirmed by acetylation [**1a**↦**1b**].

The mass spectrum of combretastatin B-1 gave a relatively small molecular ion at *m/z* 334 and two major fragment ions at *m/z* 181 ($C_{10}H_{13}O_3$) and 153 ($C_8H_9O_3$) resulting from cleavage of the benzyl bond. Results of the ms analysis suggested the presence of three methoxyl groups in one aromatic ring and a methoxyl and two hydroxyl groups in the other aromatic ring. The relationship to combretastatin A-1 was easily established by selective catalytic hydrogenation of the A-1 to B-1 [**3**]. With the relationship of combretastatin A-1 to B-1 established, examination of the ^{1}H-nmr spectrum of combretastatin B-1 was very helpful and revealed absence of the two proton doublets at δ 6.453 and 6.523. With the relatively large coupling constant and introduction of a 4-proton multiplet at δ 2.851 typical of the benzyl protons of a bibenzyl (dihydrostilbene), combretastatin A-1 [**1a**] was assumed to be a stilbene.

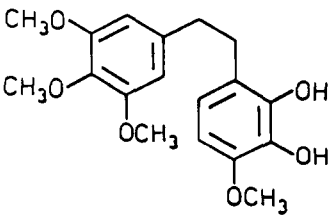
Interpretation of the ^{13}C-nmr spectrum (Table 1) of combretastatins A-1 and B-1 suggested each contained a 3,4,5-trimethoxyphenyl ring on the basis of chemical shift additive rules (8,9). In the other aromatic ring the position of the two carbons with pro-



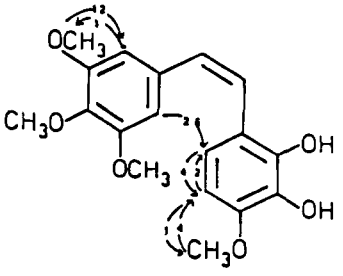
1a $R_1=R_2=H$
1b $R_1=R_2=COCH_3$
1c $R_1=R_2=Si(CH_3)_2C(CH_3)_3$



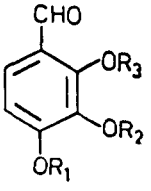
2a $R_1=R_2=H$
2b $R_1=R_2=COCH_3$
2c $R_1=R_2=Si(CH_3)_2C(CH_3)_3$



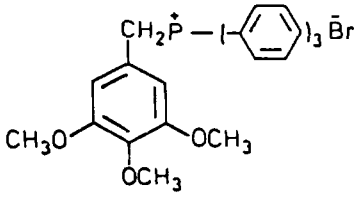
3



4 (% nOe)



5a $R_1=R_2=R_3=H$
5b $R_1=CH_3$, $R_2=R_3=H$
5c $R_1=CH_3$ $R_2=R_3=COCH_3$
5d $R_1=CH_3$, $R_2=R_3=Si(CH_3)_2C(CH_3)_3$



6

ton substituents was readily established, but the hydroxyl vs. methoxyl substituent arrangement was ambiguous. Eventually the substitution pattern in both aromatic rings was established as shown for combretastatin A-1 [**1a**] and B-1 [**3**] by application of nuclear Overhauser effect difference spectroscopy (NOEDS) methods. The most important observation here resulted from irradiation of the methoxy group at δ 3.770 resulting in a 4.3% enhancement of the ring-proton doublet at δ 6.310 (see structure **4**). Other results from the nOe experiments have been entered on structure 4.

The remaining uncertainty in completely assigning the structure of combretastatin A-1 on the basis of spectral evidence resided with the bridging olefin proton coupling constant $J=12.2$ Hz. Such coupling constants fall in the range of 6-12 Hz for *cis* protons and 12-18 Hz for *trans* protons with 10 and 17 Hz being typical values (9). While phenolic plant constituents of the stilbene type are generally isolated as the *trans*-isomers (such as from *Eucalyptus* species) (10), wood of the emetic (1) *Schotis brachypetala*, Sond (Leguminosae) (11-13) has been shown to contain a pentahydroxy *cis*-stilbene. More recently *Rheum rhaponticum* L. (Polygonaceae) (14), the commercial rhubarb, has been found to contain five *cis*-stilbenes and fourteen of the *trans* isomers.[7] In the *Rheum*

[7]Interestingly, *Rheum rhaponticum* and five other *Rheum* species have been used for a variety of primitive cancer treatments ranging from liver to breast and *Rheum officinale* has been used in China for treatment of external and cervical cancer: J.L. Hartwell, "Plants Used Against Cancer," *Quarterman Publ., Inc.*, Lawrence, Mass., 1982, p. 479.

TABLE 1. Combretastatin A-1 [**1a**] and B-1 [**3**] ^{13}C-nmr (100 MHz) Chemical Shift Assignments Relative to TMS in $CDCl_3$ Solution

Carbon	Compounds	
	1a	**3**
1	132.49[a]	138.18
2	106.13	105.67
3	152.80	153.05
4	132.67[a]	132.35
5	152.80	153.05
6	106.13	105.67
1a	130.21[b]	36.49[b]
1'a	124.06[b]	31.82[b]
1'	117.91	121.55
2'	141.72	142.19
3'	137.42	136.21
4'	146.37	145.40
5'	102.98	102.52
6'	120.17	120.32
3,5-OCH_3	55.85	56.12
4-OCH_3	60.79	60.18
4'-OCH_3	56.16	56.18

[a,b]Assignments may be interchanged.

stilbene study (14) a comparison of otherwise identical *cis*- and *trans*-stilbenes was possible, and the *cis*-olefin proton coupling constants were found to be 12 Hz and the *trans* 16 Hz. These values correspond well with those later recorded in this investigation as a result of the total synthesis summarized in the sequel. Before this information became available for interpreting the significance of the combretastatin A-1 coupling constant of 12.2 Hz, the structure was unequivocally established by an X-ray crystal structure elucidation.

The crystal structure of combretastatin A-1 [**1a**] was solved by direct methods using a SHELX-84[8] computer technique combined with refinement and difference syntheses based on SHELX-76 (15). The molecular parameters were established using the program PARST (16) and the molecular representation (Figure 1) using PLUTO (17). Combretastatin A-1 was obtained as plates in the monoclinic crystal system with space

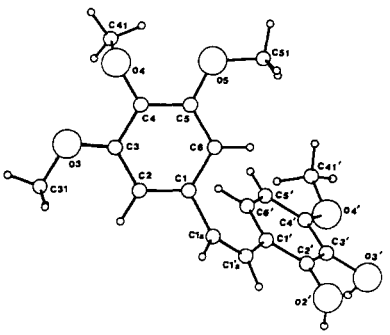


FIGURE 1. Crystal structure (PLUTO representation) of combreastatin A-1 [**1a**]

[8]G.M. Sheldrick, SHELXS-84 Direct Methods, 1983, personal communication.

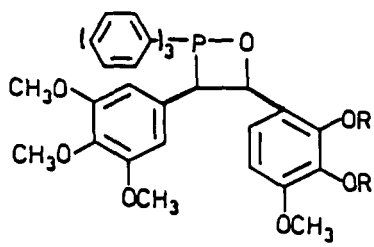
group P.2.1c. Bond lengths and angles were found to be the expected order of magnitude. The *cis*-olefin geometry was confirmed by the torsion angle C(1)-C(1a)-C(1'a)-C(1') at −6(1)°. Normals to the least-squares planes of the two phenyl rings were found inclined at 66.3(2)° to each other, and this distortion from an overall planarity of the molecule was further evidenced by the deviation from zero of the three torsion angles C(6')-C(1')-C(1'a)-C(1a) at −16(1)°, C(1')-C(1'a)-C(1a)-C(1) at −6(1)°, and C(1'a)-C(1a)-C(1)-C(6) at −58(1)°. Most likely this results from the strong steric interaction in a single molecule between C(1) . . . C(6') of 3.372(8) Å and C(6) . . . C(6') of 3.273(9) Å. Close contacts between O(2') . . . O(4), 3.242(6) Å, O(2') . . . O(5), 2.924(6) Å and O(3') . . . O(3), 3.211(6) Å are indicative of an intermolecular hydrogen bonding network. With results of the crystal structure analysis in hand, the spectral analyses were firm, including 2.6% nOe enhancement of the proton at C-6' following irradiation of the C-2 proton: a result consistent with *Z*-geometry. The stage was then set for total synthesis.

In order to obtain larger quantities of combretastatin A-1 [**1a**] for further biological evaluation, an efficient synthesis was devised based on condensing protected aldehyde **5d** with the ylide derived from phosphonium salt **6**. The important intermediate benzaldehyde **5b** required development of an improved synthesis (18,19). A selection of approaches to prepare **5b** utilizing other available starting material proved inefficient, and instead 2,3,4 trihydroxybenzaldehyde [**5a**] proved to be a most effective starting substance. Reaction of phenol **5a** with sodium borate in H_2O was found to selectively form the 2,3-borate ester, and this allowed specific methylation of the 4-hydroxyl group by dimethylsulfate. Acid hydrolysis of the borate ester afforded dihydroxybenzaldehyde **5b** which was more suitably reprotected by conversion to the 2,3-*t*-butyldimethylsilyl ether **5d** (20). Because of opinion differences in the earlier literature regarding melting points for benzaldehyde **5b**, it seemed necessary to provide some additional evidence for the structure. For this prupose benzaldehyde **5b** was acetylated, and the resulting diacetate [**5c**] was subjected to nmr irradiation of the methoxy signal at δ 3.927 and an NOEDS experiment led to 5.3% enhancement of the C-5 proton doublet at δ 6.982, thereby confirming the methoxy group at position 4.

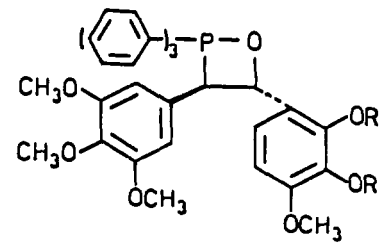
The phosphonium bromide **6** was readily prepared via 3,4,5-trimethoxybenzyl alcohol, and the corresponding ylide (prepared in THF using butyl lithium) was allowed to react with benzaldehyde **5d**. The product was a mixture of olefins **1c** and **2c** in 92.5% yield with a *Z*/*E* ratio of 9:1 by ^{1}H-nmr analysis. The *Z*-isomer (75%) was isolated by recrystallization from EtOH. Complete recovery of the remaining *Z*-isomer and the *E*-isomer on a preparative scale, either as the silyl ether derivatives or as the parent phenols, proved difficult but was readily accomplished using the diacetate derivatives. So the mixture of *Z*/*E* silyl ethers was treated with tetrabutylammonium fluoride to cleave the silyl protecting groups, and the phenols [**1a** and **2a**] were acetylated and separated by Si gel chromatography to provide combretastatin A-1 diacetate [**1b**] and its *trans* counterpart diacetate **2b**. Cleavage of disilylether **1c** with tetrabutylammonium fluoride and deacetylation of diacetate **1b** with K_2CO_3 in MeOH afforded combretastatin A-1 identical with the natural product [**1a**].

The 9:1 *Z*/*E* isomer ratio resulting from the Wittig reaction between benzaldehyde **5d** and the ylide corresponding to phosphonium bromide **6** requires comment. In the past it appeared that the oxaphosphetanes resulting from reaction of triphenylphosphonium alkylids and aldehydes were thermodynamically more stable in the *threo* configuration when prepared in the presence of lithium salts. The *threo* oxaphosphetane would then be expected to give predominantly the corresponding *trans* olefin (21). In a "salt-free" solution the oxaphosphetane was expected to have the *erythro* configuration leading to a *cis* olefin (24-26). However, recently Schlosser and Schaub (22,23) have

shown that the stereochemical environment around the group contributed by the ylide is of prime importance. Under "salt-free" conditions using (triethylphosphonio)-ethylide in THF reaction with aldehydes gave high yields of *trans* olefins. In the Wittig reaction employed to prepare combretastatin A-1 the presence of lithium bromide was obviously unimportant compared to formation of an *erythro* [7] oxaphosphetane in the most stable configuration. The sterically large silyl protecting groups probably enhance the configuration of *erythro* over the preferred *threo* [**8**]. Since ^{1}H-nmr analysis of the crude Wittig reaction product showed a *Z*/*E* ratio of 9:1, it appears likely that configuration of the intermediate oxaphosphetane was locked in place by steric effects and that little if any steric "stereochemical drift" (27) occurred between oxaphosphetane formation and production of the *cis*-olefin **1c**. To evaluate the preceding hypothesis, the course of the Wittig reaction was studied using ^{31}P nmr (61.99 MHz), and the results (cf. experimental) clearly showed that there was no detectable *cis-trans* interconversion.



erythro **7**
R=$Si(CH_3)_2C(CH_3)_3$



threo **8**
R=$Si(CH_3)_2C(CH_3)_3$

Preliminary biological evaluation of the *cis* and *trans* olefins against the PS cell line gave some interesting insights into structural requirements for cell growth inhibitory activity. Combretastatin A-1 diacetate [**1b**] was found to be three-fold less active at PS ED_{50} 2.7 μg/ml than the parent natural product [**1a**]. The *trans*-isomer counterpart **2b** was essentially inactive with PS ED_{50} 12 μg/ml. The silyl ether derivatives **1c** and **2c** were also inactive against the PS cell line.

The previously reported active compound isolated from *C. caffrum*, combretastatin, (5,6) was found to cause mitotic arrest in cells in culture (28,29) and to interact with tubulin, the major protein component of microtubules (28,29). Since the newly characterized *C. caffrum* natural products [**1a** and **3**] are structurally similar to the initial compound, they were evaluated for in vitro interactions with tubulin (Table 2). They were compared to combretastatin and to three additional well-characterized antimitotic agents with structural analogies to the combretastatin family, colchicine, podophyl-

TABLE 2. Inhibition of Microtubule Assembly and Binding of Colchicine to Tubulin by Combretastatin A-1 [**1a**] and Combretastatin B-1 [**3**]

Drug	Experiment I Microtubule Assembly ID_{50}[a] (μM)	Experiment II Colchicine Binding % of control
Combretastatin A-1 . . .	2	2.2
Combretastatin B-1 . . .	3	13
Combretastatin	11	34
Podophyllotoxin	3	13
Steganacin	6	49
Colchicine	6	—

[a]Defined as the drug concentration inhibiting the extent of microtubule assembly by 50%.

lotoxin, and steganacin, all of which bind at a common site on tubulin (30-34). Combretastatin A-1 was more active than combretaastatin B-1 in its interactions with tubulin, in agreement with its greater antineoplastic activity; both compounds were significantly more potent than the previously described combretastatin (5,28,29). In microtubule assembly (Experiment I), equivalent inhibition was observed with 2 μM combretastatin A-1, 3 μM combretastatin B-1, and 11 μM combretastatin. The inhibition of assembly with combretastatins A-1 and B-1 was comparable to that observed with podophyllotoxin and greater than that observed with colchicine and steganacin.

Combretastatin, podophyllotoxin, steganacin, and colchicine all appear to bind at the same site on tubulin, as the first three agents act as competitive inhibitors of the binding of radiolabeled colchicine to the protein (28-32). Combretastatins A-1 and B-1 were particularly potent as inhibitors of the binding of [^{3}H]colchicine to tubulin (Table 2, Experiment II), significantly exceeding the inhibition observed with steganacin, combretastatin and even, in the case of combretastatin A-1, podophyllotoxin.

Further studies of combretastatins A-1 and B-1 are in progress.

EXPERIMENTAL

Synthetic intermediates were employed as received from Sigma-Aldrich. Solvents used for chromatographic procedures were redistilled. The Sephadex LH-20 (particle size 25-100 μm) used for steric exclusion chromatography was obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Si gel 60 (70-230 mesh) utilized for column adsorption chromatography and the Lobar Si gel 60 columns (size B) were supplied by E. Merck (Darmstadt, Germany). Si gel GHLF Uniplates (0.25 mm layer thickness) were obtained from Analtech, Inc., (Newark, Delaware). The thin layer chromatograms were developed with anisaldehyde-HOAc or ceric sulfate-H_2SO_4 spray reagents and examined after heating at approximately 150° for 5-10 min, or under uv light.

In all synthetic procedures solvent extracts of aqueous solutions were dried over anhydrous Na_2SO_4. All melting points were uncorrected and were determined using a Kofler-type hot-stage apparatus. Uv spectra were recorded using a Hewlett-Packard Model 8540A UV/VIS spectrophotometer and ir spectra with a Nicolet Ft-IR Model MX-1 instrument. Nmr experiments were conducted with Bruker WH-90 and AM-400 instruments with $CDCl_3$ as solvent and TMS as internal standard. Chemical shifts are recorded using the δ scale. The nOeds experiments were performed (Dr. R.A. Nieman) with a $CDCl_3$ solution degassed six times by the freeze-thaw technique. Mass spectral deteriminations were made with a MS-50 instrument at the NSF Regional Facility, University of Nebraska, Lincoln, Nebraska. Elemental microanalyses were performed at Mic-Anal, Tucson, Arizona. The X-ray crystal structure determination was performed with an Enraf-Nonius CAD-4 diffractometer, and all computations were performed using a Sperry 1100 computer.

PLANT TAXONOMY.—Stem wood of the South African tree *C. caffrum* was collected and identified as part of the National Cancer Institute-U.S. Department of Agriculture research program directed by Drs. John D. Douros, Matthew I. Suffness, and James A. Duke. The stem wood (B817373) employed in this study was obtained in 1979.

EXTRACTION AND SOLVENT PARTITION PROCEDURES..—The dry stem wood (77 kg) of *C. caffrum* was subdivided by chipping and extracted with CH_2Cl_2-MeOH (1:1, 320 liters) at ambient temperature for 11 days. The CH_2Cl_2 phase was separated by addition of H_2O (25% by volume), and the plant extraction was repeated with another 320 liters of CH_2Cl_2-MeOH (1:1) as just described. The combined CH_2Cl_2 phases were concentrated to a crude extract weighing 1.42 kg and showing PS in vivo life extension of 27% at 100 mg/kg and PS ED_{50} 5.1 μg/ml. A solution of the CH_2Cl_2 fraction was partitioned 5× between hexane (18 liters) and MeOH-H_2O (9:1, 18 liters). After separating the hexane phase the MeOH-H_2O was adjusted to a concentration of 3:2 and extracted (5×) with CH_2Cl_2 (18 liters). The hexane extract (602.3 g) proved PS in vivo inactive and marginally active against the cell line with ED_{50} 2.4 μg/ml. The PS in vivo activity (38-41% life extension at 25-50 mg/kg) and major cell growth inhibition (ED_{50} 0.21 μg/ml) was concentrated in the CH_2Cl_2 fraction (827.9 g) from the solvent partitioning sequence.

ISOLATION OF COMBRETASTATINS A-1 [**1a**] AND B-1 [**3**].—The CH_2Cl_2 fraction from the solvent partitioning sequence was dissolved in MeOH (7×500 ml) and further separated by steric exclusion chromatography on columns of Sephadex LH-20 (7×2.5 kg). The PS active (41% life extension at 12.5 mg/kg and ED_{50} 0.18 μg/ml) fraction (30.6 g) was further separated in hexane-toluene-MeOH (3:1:1) solution by partition chromatography on Sephadex LH-20 (2.5 kg). Further concentration of the active com-

ponents was achieved by this important separation step that gave a fraction (8.11 g) with 30-48% life extension at 12.5-50 mg/kg and ED_{50} 2.7 μg/ml in the bioassay. The 8.11 g active fraction was chromatographed in EtOAc (20 ml) on a column of Si gel (200 g). Elution with hexane-EtOAc (3:1) led to two active fractions weighing 0.64 g and 2.25 g. Recrystallization of the 2.25 g fraction from hexane/$CHCl_3$ afforded a pure specimen of combretastatin A-1 [**1a**] (0.70 g, 9.1×10^{-1}% yield based on the dried plant) as plates melting at 113-115°: uv (MeOH) λ max 233, 255, 298 mμ (ε 7145, 7766, 7848; uv (MeOH+MeONa) λ max 232, 255, 288, 397 mμ (ε 7323, 7679, 7038, 1983); ir (film) 3482, 3426, 1580, 1507, 1480, 1463, 1452, 1328, 1290, 1238, 1125, 1092, 1000, 915, 850 cm^{-1}; ^1H nmr (400 MHz) 3.597 (6H, s, 2× OCH_3-3,5), 3.760 (3H, s, OCH_3-4), 3.770 (3H, s, $OCH_3$4′), 5.438 (2H, br s, disappeared upon D_2O exchange 2×OH-2′,3′), 6.310 (1H, d, J_{AB}=8.64 Hz, H-5′), 6.453 (1H, d, $J_{A'B'}$=12.2 Hz, -CH=CH-), 6.460 (2H, s, H-2,6), 6.523 (1H, d, $J_{B'A'}$=12.2 Hz, -CH=CH-), 6.691 (1H, d, J_{BA}=8.6 Hz, H-6′); ^{13}C nmr (see Table 1); hreims *m/z* 332.1248 (M^+, 100%, calcd 332.1259 for $C_{18}H_{20}O_6$) and 317.1005 (m^+-CH_3, 93.7%, $C_{17}H_{17}O_6$).

Anal. calcd for $C_{18}H_{20}O_6$: C, 65.05; H, 6.06. Found: C, 64.80; H, 6.08.

The 0.6 g active fraction from the Si gel column chromatograph was rechromatographed using two Lobar B columns in series. Elution with hexane-EtOAc (7:3) provided combretastatin B-1 as an oil (39.6 mg) in 5.1×10^{-5}% yield based on the dry plant starting material. The colorless, gummy combretastatin B-1 [**3**] exhibited uv (MeOH) λ max 239, 270 mμ (ε 5845, 1949); uv (MeOH+MeONa) λ max 240, 256 mμ (ε 5860, 5949); ir (film) 3424, 3408, 1590, 1508, 1457, 1288, 1126, 1093 cm^{-1}; ^1H nmr (400 MHz) 2.851 (4H, m, -CH_2-CH_2-), 3.827 (3H, s, OCH_3-4′), 3.831 (6H, s, 2× OCH_3-3,5), 3.856 (3H, s, OCH_3-4), 5.382 5.398 (1H each, D_2O exchangeable, 2× OH-2′,3′), 6.390 (1H, d, J_{AB}=58.36 Hz, H-5′), 6.420 (2H, s, H-2,6), 6.577 (1H, d, J_{BA}=8.36 Hz, H-6′); ^{13}C nmr (refer to Table 1); hreims *m/z*, 334.1417 (27.2%, M^+, calcd, $C_{18}H_{22}O_6$ for 334.1416) 181.0861 (100, calcd $C_{10}H_{13}O_3$ for 181.0865) and 153.0549 (59.6 calcd $C_8H_9O_3$ for 153.0552).

ACETYLATION OF COMBRETASTATIN A-1 [**1a**].—A solution of combretastatin A-1 (5 mg) in 0.5 ml of Ac_2O-pyridine (1:1) was allowed to stand overnight at room temperature. The volatile components were evaporated under a stream of N_2 and the product crystallized from hexane/EtOAc to afford colorless plates of acetate **1b**: mp 133-135°; ir (film) 1775, 1579, 1503, 1454, 1420, 1206, 1174, 1127, 1088, 1010 cm^{-1}; ^1H nmr (400 MHz) 2.264, 2.299 (3H each, s, $COCH_3$), 3.664 (6H, s, 2× OCH_3), 3,807 (3H, s, OCH_3) 3.813 (3H, s, OCH_3), 6.361 (1H, d, J_{AB}=11.90 Hz, -CH=CH-), 6.442 (2H, s, H-2,6), 6.548 (1H, d, J_{AB}=11.90 Hz, -CH=CH-), 6.726 (1H, d, $J_{A'B'}$=8.7 Hz, H-5′), 7.025 (1H, d, $J_{A'B'}$=8.7 Hz, H-5′), 7.025 (1H, d, $J_{B'A'}$=8.7 Hz, H-6′); hreims *m/z* 416.1463 (60 M^+, calcd $C_{22}H_{24}O_8$ for 416.1471), 374.1363 (70, $M+H)^+$-$COCH_3$, $C_{20}H_{22}O_7$) and 332.1263 (100, $(M+2H)^+$-2× $COCH_3$, $C_{18}H_{18}O_6$).

COMBRETASTATIN B-1 [**3**] BY HYDROGENATION OF COMBRETASTATIN A-1 [**1a**].—A mixture of combretastatin A-1 (35 mg) in MeOH (15 ml) and 5% Pd/C (10 mg) was treated with a positive pressure of H_2 at ambient temperature overnight. Catalyst was removed by filtering the yellow solution, and the product was purified by preparative layer chromatography on Whatman Si gel plates with Me_2CO-CH_2Cl_2 (2:23) as mobile phase. The product was identical (by tlc, ir, and nmr) with natural combretastatin B-1.

THE CRYSTAL AND MOLECULAR STRUCTURE OF COMBRETASTATIN A-1 [**1a**].—Single crystals of combretastatin A-1 [**1a**] were obtained from hexane/$CHCl_3$. The crystals were small, very thin plates and as such not entirely suitable for X-ray analysis. However, one such crystal was selected for irradiation. During the data collection, intensities of three standard reference reflections were monitored every hour, and centering was checked every 100 measured reflections. Intensities were corrected for Lorentz and polarization effects but not for absorption. The structure was solved by direct methods using a preliminary version of SHELXS-84[8], which yielded in an E map. 23 of the 24 non-hydrogen atoms. Subsequent refinement and difference syntheses using SHELX-76 (15) enabled location of the remaining non-hydrogen atom. Hydrogen atoms of the phenyl rings and the olefinic group were placed in calculated positions with a single temperature factor. Methyl hydrogens were treated as rigid groups with a single temperature factor. The two hydroxyl hydrogens were initially placed as located in a difference map and constrained to ride at 1.00 A from their parent oxygens. In the final refinements all atoms were treated with isotropic thermal motion. Molecular parameters were obtained using PARST (16) and a drawing of the molecule using PLUTO (17). Further details of the data collection, solution, and refinement of the structure are given in Table 3. Final atomic coordinates of the molecule are shown in Table 4,[9] and a perspective view with atomic nomenclature is given in Figure 1.

[9]Atomic coordinates for the structure have been deposited with the Cambridge Crystallographic Data Centre and can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, Lensfield Rd., Cambridge, CB2 1EW, UK.

TABLE 3. Crystallographic Data and Summary of Intensity Data Collection and Structure Refinement for Combretastatin A-1 (**1a**)

Molecular formula	$C_{18}H_{20}O_6$
Mr. g mol	332.35
Crystal system	monoclinic
Space group	$P2_1/c$
T, K	294
a, Å	10.497(2)
b, Å	6.717(2)
c, Å	22.746(4)
β, o	96.11(2)
V, Å3	1594.7(6)
Z	4
d calc, g cm^{-3}	1.38
Crystal dimensions, mm	0.06×0.16×0.34
Radiation wavelength MoK α, Å	0.7107
Crystal decay, %	<1
μ, cm^{-1}	0.972
F(000)	704
Scan mode	ω-2θ
Scan width in ω, °	(0.64+0.35 tan θ)
Aperture width, mm	(1.12+1.05 tan θ
Aperture length, mm	4
Final acceptance limit	20 τ at 20° min
Maximum recording time, s	40
Scan range, 2θ	2-46
No. of reflections collected	1793
No. of reflections observed (with Irel> 2σ Irel)	1265
No. of parameters:	
$R=\Sigma\|\|Fo\|-\|Fc\|\|/\Sigma\|Fo\|$	0.076
$R_n=\Sigma_n ½\|\|Fo\|-\|Fc\|\|/\Sigma w ½\|Fo\|$	0.074
W	(σ^2F)-1

SYNTHESIS OF 2,3-DIHYDROXY-4-METHOXY-BENZALDEHYDE [**5b**].—To a vigorously stirred solution of sodium borate-decahydrate (borax, 30 g) in 600 ml of H_2O was added 2,3,4-trihydroxy-benzaldehyde [**5a**] (5 g, 32.4 mmol). The yellow solution was stirred at room temperature for 30 min followed by dropwise and simultaneous addition (over 30 min) of NaOH (4.0 g, 100 mmol) in H_2O (50 ml) and dimethylsulfate (9.45 ml, 100 mmol). Vigorous stirring was continued overnight and conc. HCl was added to pH 1. After stirring for an additional 30 min, the mixture was extracted with $CHCl_3$ (5× 300 ml). The organic layer was once washed with brine, dried, and evaporated to yield a slightly yellowish solid which on crystallization from EtOAc/hexane afforded slightly yellowish colored needles [**5a**] (3.9 g, 72%), mp 116-117°: [lit. (19) 118-119°]; ir (film) 3374, 1646, 1624, 1505, 1461, 1443, 1278, 1210, 1106, 636 cm^{-1}; ^{1}H nmr 3.987 (3H, s, OCH_3), 5.466 (1H, brs. OH-3, D_2O exchanged), 6.617 (1H, d, J_{AB}=8.62, H-5), 7.147 (1H, d, J_{BA}=8.62 Hz, H-6), 9.757 (1H, s, CHO) 11.113, (1H, brs. OH-2, D_2O exchanged); hreims *m/z* 168.0419 (M^+, 100%, calcd 168.0423 for $C_8H_8O_4$).

Diphenol **5b** (100 mg) was acetylated with Ac_2O-pyridine (see above) to afford diacetate **5c** as crystals from Me_2CO/hexane: mp 126.5-128.5°; ir (film) 1772, 1693, 1609, 1506, 1459, 1370, 1295, 1202, 1174, 1101, 807 cm^{-1}; ^{1}H nmr (400 MHz) 2.331 (3H, s, $COCH_3$), 2.386 (3H, s, $COCH_3$), 3.927 (3H, s, OCH_3), 6.982 (1H, d, J_{AB}=8.8 Hz, H-5), 7.749 (1H, d, J_{BA}=8.8 Hz, H-6), 9.907 (1H, s, CHO); hreims *m/z* 210.0524 (M^+, 20%, calcd for 210.0528 $C_{10}H_{10}O_5$), and 168.0417 (100% $[M+H]^{\pm}COCH_3$, $C_8H_8O_4$).

Anal. calcd for $C_{12}H_{12}O_6$, C, 57.15; H, 4.80; Found: C, 57.18; H, 4.75.

3,4,5-TRIMETHOXY-BENZYLTRIPHENYLPHOSPHONIUM BROMIDE [**6**].—A solution of triphenylphosphine (4.2 g) in toluene (10 ml) was added to a stirred solution of 3,4,5-trimethoxybenzyl bromide (4.0 g) in toluene (15 ml), and stirring was continued for 24 h. The phosphonium bromide that separated (8.0 g, 99%) was collected and dried under vacuum, mp 223-224° [lit. (35) 222-223°].

TABLE 4. Fractional Atomic Coordinates ($\times 10^4$) and Temperature Factors ($Å^2 \times 10^3$) for Non-hydrogen Atoms of Combretastatin A-1 **[1a]**

	x/a	y/b	z/c	U_{iso}
C(1)	6517(6)	4976(9)	1646(3)	37(2)
C(2)	6985(6)	6387(10)	1286(3)	36(2)
C(3)	8212(6)	6195(9)	1116(3)	37(2)
O(3)	8750(4)	7487(7)	742(2)	50(1)
C(31)	7995(7)	9107(10)	512(3)	52(2)
C(4)	8988(6)	4633(9)	1319(3)	32(2)
O(4)	10241(4)	4564(6)	1183(2)	42(1)
C(41)	10442(7)	3270(11)	711(3)	57(2)
C(5)	8514(6)	3209(9)	1675(3)	33(2)
O(5)	9352(4)	1693(6)	1862(2)	39(1)
C(51)	8917(6)	157(10)	2230(3)	42(2)
C(6)	7286(5)	3361(10)	1845(3)	34(2)
C(1a)	5250(6)	5280(10)	1852(3)	42(2)
C(1'a)	4222(6)	4122(9)	1822(3)	38(2)
C(1')	3959(5)	2190(9)	1519(3)	34(2)
C(2')	2868(5)	1124(9)	1638(3)	34(2)
O(2')	2148(4)	1839(7)	2063(2)	45(1)
C(3')	2509(6)	−621(9)	1350(3)	34(2)
O(3')	1431(4)	−1586(7)	1510(2)	51(1)
C(4')	3198(5)	−1314(9)	916(3)	32(2)
O(4')	2702(4)	−3024(7)	635(2)	47(1)
C(41')	3459(7)	−3992(11)	238(3)	57(2)
C(5')	4285(6)	−324(9)	780(3)	36(2)
C(6')	4648(6)	1401(9)	1085(3)	38(2)

2,3,-BIS-[(TERT-BUTYLDIMETHYLSILYL)-OXY]-4-METHOXY-BENZALDEHYDE **[5d]**.—Diisopropylethylamine (1.6 ml, 9.0 mmol) was added to a stirred solution (under argon) of 2,3-dihydroxy-4-methoxybenzaldehyde (0.50 g, 2.98 mmol) in DMF (5 ml) followed by *t*-butyldimethylsilyl chloride (1.0 g, 6.66 mmol). The reaction mixture was stirred at room temperature for 20 min. Ice (10 g) was added, and the mixture was extracted with Et_2O (3×15 ml). The ethereal solution was washed with H_2O (15 ml), saturated $NaHCO_3$ solution (2×10 ml), H_2O (20 ml), and solvent evaporated to yield silyl ether **5d** as a chromatographically homogeneous oil (1.15 g, quantitative) that crystallized from MeOH: mp 74.5-76°; ir (film) 2931, 1684, 1586, 1454, 1292, 1264, 1099, 843, 827 cm^{-1}; ^1H nmr, 0.132 (12 H, s, 4× $SiCH_3$), 0.987 (9 H, s, 3× CH_3), 1.038 (9 H, s, 3× CH_3), 6.612 (1 H, d, J_{AB}=8.7 Hz, H-5), 7.483 (1 H, d, J_{BA}=8.7 Hz, H-6), 10.225 (1H, s, CHO); hreims *m/z* 381.1915 (5, M^+ CH_3, calcd 381.1917 for $C_{19}H_{33}O_4Si_2$), 339.1429 (100, M^- C_4H_9, calcd 339.1448 for $C_{16}H_{27}O_4Si_2$).

Anal. calcd for $C_{20}H_{36}O_4Si_2$, C, 60.56; H, 9.15. Found: C, 60.38; H, 9.28.

2',3'-BIS-[(*T*-BUTYLDIMETHYLSILYL)-OXY]-(*Z*) AND (*E*)-COMBRETASTATIN A-1 **[1c AND 2c]**.—*Synthetic Procedure.*—Butyllithium (20 ml, 1.5 M in hexane, 30 mmol) was added (under argon) to a suspension of 3,4,5-trimethoxybenzyltriphenylphosphonium bromide **[6]** (15.7 g, 30 mmol) in THF (450 ml) at −15°. The resulting deep reddish solution was allowed to stir at room temperature for 30 min. Aldehyde **5d** (11.09 g, 28.0 mmol) was added, and the reaction mixture was diluted with ice-cold H_2O and extracted with Et_2O (3× 250 ml). The ethereal solution was washed with H_2O, and solvent was evaporated to yield a crude product that was crystallized from EtOH to afford pure *Z*-isomer **1c** (11.0 g) and a mixture (1:1, by ^1H nmr) of *Z/E* isomer (3.5 g, total yield 92.5%). The *Z*-isomer recrystallized from MeOH/EtOAc to furnish colorless needles: mp 117-118°; ir (film) 1580, 1507, 1496, 1472, 1456, 1445, 1420, 1248, 1129, 1102, 1010, 840, 780 cm^{-1}; ^1H nmr (400 MHz) 0.105 (6H, s, 2× Si-CH_3), 0.190 (6H, s, 2× $SiCH_3$), 0.999 (9H, s, 3× CH_3), 1.038 (9H, s, 3× CH_3), 3.674 (6H, s, 2× OCH_3), 3.738 (3H, s, OCH_3), 3.835 (3H, s, OCH_3), 6.358 (1H, d, $J_{A'B'}$=12.0 Hz, -CH=CH-), 6.361 (1H, d, J_{AB}=8.7 Hz H-5'), 6.584 (1H, d, $J_{B'A'}$=12.0 Hz, -CH=CH-), 6.619 (2H, s, H-2,6), 6.910 (1H, d, J_{BA}=8.7 Hz, H-6'); hreims *m/z* 560.2941 (90%, M^+, calcd 560.2989 for $C_{30}H_{48}O_6Si_2$), 488.2060 (100, $M^{\pm}$ C_5H_{12}, $C_{25}H_{36}O_6Si_2$).

Anal. calcd for $C_{30}H_{48}O_6Si_2$, C, 64.25; H, 8.63. Found: C, 64.03; H, 8.70.

A small portion of the *Z/E* mixture was chromatographed on a Si gel column and eluted with hexane-EtOAc (49:1). The fraction enriched with the *E*-isomer crystallized from MeOH/EtOAc to afford pure *E*-

isomer **2c** as colorless plates melting at 139-140°: ir (film) 1581, 1507, 1496, 1472, 1463, 1456, 1444, 1239, 1130, 1101, 840, 785 cm^{-1}; ^1H nmr (400 MHz) 0.114 (6H, s, 2× $SiCH_3$), 0.133 (6H, s, 2× $SiCH_3$), 0.999 (9H, s, 3× CH_3), 1.092 (9H, s, 3× CH_3), 3.793 (3H, s, OCH_3), 3.862 (3H, s, OCH_3), 3.884 (6H, s, 2× OCH_3), 6.556 (1H, d, J_{AB}=8.72 Hz, H-5'), 6.716 (2H, s, H-2,6), 6.805 (1H, d, $J_{A'B'}$=16.44 Hz, -CH=CH-), 7.198 (1H, d, J_{BA}=8.72 Hz, H-6'), 7.308 (1H, d, $J_{B'A'}$=16.44 Hz, -CH=CH-); hreims *m/z* 560.3151 (100, M^+ calcd for 560.2989, ($C_{30}H_{48}O_6Si_2$) 488.2059 (90, M^+-C_5H_{12}, $C_{25}H_{36}O_6Si_2$).

Anal. calcd for $C_{30}H_{48}O_6Si_2$ ½ H_2O: C, 63.23; H, 8.66. Found: C, 63.32; H, 8.49.

In another experiment, when 1.5 equivalents of *n*-butyllithium were used per equivalent of phosphonium bromide [**6**], the ratio of *Z*/*E* isomer changed dramatically from 9:1 to 3.5:1.

^{31}P-NMR EVALUATION.—Phosphonium Salt **6** (0.523 g, 1.0 mmol) in dry THF (20 ml) was treated (under argon) with 1.0 molar equivalent of *n*-butyllithium at −15° to generate the ylide. An aliquot (2.0 ml, 0.10 mmol) of the ylide solution was transfered to an nmr tube (10 mm) and frozen (liquid N_2). A solution of aldehyde **5d** (39.0 mg, 0.098 mmol) in THF-d_8 (1 ml) was added, and the frozen sample was warmed to −80° in the nmr probe. Examination of the spectrum (at −80°) showed three sharp singlets at δ24.538 (ylide), 7.553 (*cis* oxaphosphetane **7**), and δ-8.0 ppm (*trans* oxaphosphetane **8**), integrating in the ratio 15:65:1, respectively. On warming to −60° during 10 min the *cis* oxaphosphetane was formed at the expense of the ylide, and the ratio changed to 9:69:1. While disappearance of the ylide was still in progress, a new broad singlet started appearing (−50°, 10 min) at 28.4 ppm, due to formation of triphenylphosphine oxide (as a lithium bromide complex), the *trans* oxaphosphetane signal disappeared, and the ratio of signals from downfield to upfield was 11:3:64:0. The disappearance of *cis* oxaphosphetane and appearance of triphenylphosphine oxide was monitored at −30° (10 min after −50°) and −10° (12 min after −30°) to give the ratios 11:23 and 33:18, respectively. After another 12 min at 25° the oxaphosphetane disappeared completely. The results clearly indicate that there were no interconversions of *cis* to *trans* oxaphosphetanes. The 10% of *E*-isomer may have been formed due to isomerization during isolation. The shift of ^{31}P in the triphenylphosphine oxide lithium bromide complex was found to change with temperature as follows: −50° (28.4), −30° [28.0 (24) used as reference]. −10° (27.9), and 25° (26.3).

2',3'-DIACETOXY-4'-METHOXY-*Z*- AND *E*-COMBRETASTATIN A-1 [**1b, 2b**].—To a 1.8 g sample of the isomer mixture in THF (10 ml) was added tetrabutylammonium fluoride (8 ml of a 1M solution in THF), and the mixture was stirred (under argon) at room temperature for 15 min. Et_2O (50 ml) was added, the solution was washed with H_2O (2×50 ml), and the solvent was removed under reduced pressure. The residue was acetylated in 4 ml of Ac_2O-pyridine (1:1). After stirring overnight the acetylation mixture was poured into ice water, extracted with Et_2O (3×50 ml), washed successively with 1N HCl (2×25 ml), saturated $NaHCO_3$ solution (2×25 ml), and H_2O (50 ml). Removal of solvent furnished a gummy residue that was chromatographed on a column of Si gel (50 g). Gradient elution with hexane-EtOAc (9:1-1:1) afforded 0.55 g of *Z*-isomer **1b** and 0.60 g of *E*-isomer **2b**. The *Z*-isomer **1b** was recrystallized from hexane/EtOAc to give colorless prisms, mp 133-135° identical with diacetate **1b** prepared from natural combretastatin A-1 [**1a**].

Anal. calcd for $C_{22}H_{24}O_8$: C, 63.46; H, 5.81. Found: C, 63.37; H, 5.79.

The *E*-isomer **2b** recrystallized as needles (mp 172-173°) from hexane/EtOAc: ir (film) 1775, 1582, 1507, 1455, 1295, 1206, 1173, 1126, 1089, 670 cm^{-1}. ^1H-nmr (400 MHz) 2.313 (3H, s, $COCH_3$), 2.348 (3H, s, $COCH_3$), 3.863 (6H, s, 2×OCH_3), 3.897 (3H, s, OCH_3), 3.899 (3H, s, OCH_3), 6.669 (2H, s, H-2,6), 6.870 (1H, d, J_{AB}=16.02 Hz, -CH=CH-), 6.897 (1H, d, $J_{A'B'}$=8.5 Hz, H-5'), 6.917 (1H, d, J_{BA}=16.02 Hz -CH=CH-), 7.470 (1H, d, $J_{B'A'}$=8.5 Hz, H-6'); hreims *m/z* 416.1486 (33, M^+, calcd 416.1471 for $C_{22}H_{24}O_8$), 374.1347 (39, M+H$^{\pm}$$COCH_3$), 332.1234 (46, M+2H$^+$-2×$CH_3CO$).

Anal. calcd for $C_{22}H_{24}O_8$ ½ H_2O, C, 62.11; H, 5.92. Found: C, 62.27; H, 5.73.

COMBRETASTATIN A-1 [**1a**].—*Method A.*—A 60-mg sample of the synthetic diacetate **1b** in MeOH (3 ml) was stirred (under argon) with K_2CO_3 (50 mg) for 1 h. HCl (1N) was added, and the phenol was extracted with $CHCl_3$ (3×10 ml), washed with H_2O (10 ml), and solvent removed. The product was passed through a pipette of Si gel (1.0 g) to yield combretastatin A-1 [**1a**] (45 mg 94%). The viscous oil crystallized from hexane/$CHCl_3$ to afford a pure specimen **1a** as plates, mp 114-115°, identical with the natural product.

Method B.—A solution of the silyl ether **1c** (10.78 g, 19.26 mmol) in THF (100 ml under argon) was treated with tetrabutylammonium fluoride (45 ml, 1M solution in THF) and stirred for 10 min. After completion the reaction mixture was extracted with Et_2O (300 ml). The ethereal solution was washed with cold H_2O (2×200 ml), dried, and evaporated to a powder [**1a**] (6.0 g, 93.8%), which crystallized from $CHCl_3$/hexane as plates, mp 113-115°.

Anal. calcd for $C_{18}H_{20}O_6$, C, 65.06; 6.07. Found: C, 64.48; H, 6.03.

MICROTUBULE ASSEMBLY.—The assembly reaction at 37° was followed turbidimetrically as described previously (28,29). Each 0.25 ml reaction mixture contained 1.5 mg/ml of tubulin and 0.5 mg/ml of microtubule-associated proteins [proteins were purified as described elsewhere (36)], 0.1 M 4-morpholine ethanesulfonate (adjusted to pH 6.6 with NaOH), 0.5 mM $MgCl_2$, 0.5 mM guanosine 5′-triphosphate, and drugs as required. The concentration of drug needed to inhibit the extent of assembly by 50% was determined.

BINDING OF [^{3}H]COLCHICINE TO TUBULIN.—Binding of radiolabeled colchicine to tubulin was measured by retention of drug-tubulin complex on DEAE-cellulose paper filters, as described previously (28,29). Reaction mixtures (0.1 ml) contained 0.1 mg/ml of tubulin, 5 μM [^{3}H]colchicine, the competing drug at 5 μM, 1.0 M monosodium glutamate (adjusted to pH 6.6 with HCl), 0.1 M glucose-1-phosphate, 1mM $MgCl_2$, 1 mM guanosine 5′-triphosphate, and 0.5 mg/ml bovine serum albumin [the latter four components substantially enhance the rate of the reaction (37)]. Incubation was for 10 min at 37°.

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