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Synthesis and Biological Evaluation of 4-Arylcoumarin Analogues of Combretastatins. Part 2

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ABSTRACT: A series of A-ring variously methoxylated 4-(3-hydroxy-4-methoxyphenyl)coumarins related to combretastatin A-4 was prepared by cross-coupling reactions. Cytotoxicity studies indicated a potent activity against HBL100 cell line. Substitution patterns on A-ring had only a slight effect on antiproliferative activity. For most cytotoxic compounds, the activity as potential modulators of P-gp and BCRP efflux pumps was evaluated. The results show that compounds **2** and **7** were able to restore mitoxantrone accumulation (BCRP) at concentrations similar to that of cyclosporine A. Compound **7** was the most efficient to reverse P-gp activity. All compounds were found to potently inhibit in vitro microtubule formation via a substoichiometric mode of action for the most part. Compounds **1** and **2** were found to have an apparent affinity binding constant similar to that of combretastatin A-4, i.e., 1×10^6 M^{-1}. The molecular modeling of coumarin derivatives was performed on the basis of the molecular structure of **7**, as determined by single-crystal X-ray crystallography. The calculations suggested that the presence of a methoxy group out of the plane of the chromenone moiety is an important steric hindrance factor embedding the accessibility of those molecules inside the binding pocket on tubulin.

INTRODUCTION

The microtubule network is an essential component of the cytoskeleton of eukaryotic cells. The discovery of the binding of colchicine to tubulin was a fundamental step in the development of antimitotic drugs (Chart 1). The development of new antimitotic drugs then led to new cancer chemotherapy approaches and to a better knowledge of microtubule pharmacology and biochemistry. Antimitotic drugs that bind tubulin or antitubulin agents are classified as microtubule-stabilizing agents or microtubule-destabilizing agents. The taxoids belonging to the first class and the vinca alkaloïds belonging to the second one are commonly used in cancer therapy.[1] Considering the success of the antitubulin pharmacological class, numerous studies are now focused on the discovery and clinical trial development of new derivatives. Among them, combretastatin A-4 (CA-4), isolated from the bark of the *Combretum Caffrum* tree, has been shown to be a potent tubulin assembly inhibitor and vascular disrupting agent at low concentrations.[2]

At the present time, due to the relative structural simplicity of combretastatin A-4, hundreds of combretastatin derivatives have been synthesized and studied. Among them, several candidates are in advanced clinical trials, such as combretastatin A-4 phosphate (CA-4P), combretastatin A1 diphosphate (OXi 4503), and combretastatin AVE 8062A.[3−6] It is too early to predict if any of these combretastatin derivatives will enter clinical use, and research to develop new analogues with a more favorable therapeutic window is ongoing. In the authors' laboratories, the synthesis of different 4-arylcoumarin analogues of combretastatin A-4 led to the identification of two new compounds **7** and **8** (Chart 1) with potent cytotoxic activity on a CEM leukemia cell line.[7] It was suggested that the cytotoxicity of compounds **7** and **8** may be related to their interaction with microtubules and tubulin because these compounds, previously synthesized as potential topoisomerase inhibitors, inhibit microtubule formation from purified tubulin.

These compounds disturbed cell survival by depolymerizing the microtubule network, leading to a mitotic block.[8] By isothermal titration calorimetry (ITC), it was demonstrated using a displacement method that combretastatin A-4 and 4-arylcoumarins share their binding domain in tubulin. A superimposition of the combretastatin A-4 and these analogues on the tubulin colchicine binding domain indicated that the hydroxyl group present on the B-ring of compounds **7**, **8**, and combretastatin A-4 could form a

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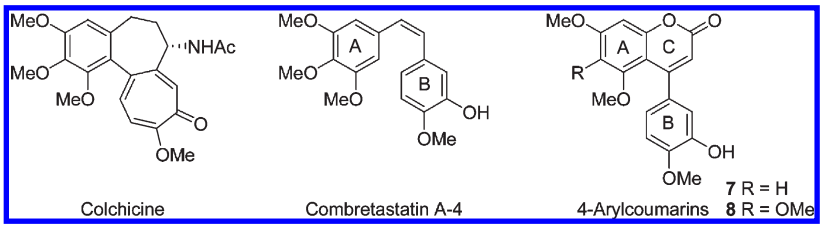
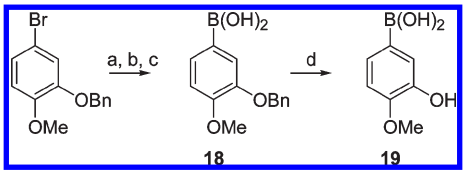
Chart 1. Structural Analogy of Compounds That Inhibit Tubulin Assembly

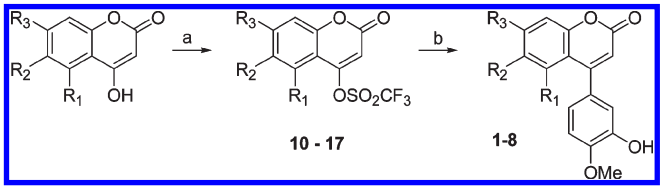


Scheme 1. Synthesis of 3-Hydroxy-4-methoxyphenylboronic Acid 19[a]



[a] (a) *n*-BuLi, THF, −78 °C; (b) $(i\text{-PrO})_3B$, −78 °C; (c) HCl_{aq}; (d) H_2, Pd/C, THF.

Scheme 2. Synthesis of a Series of 4-Arylcoumarin Analogues of Combretastatin A-4, 1−8[a]



[a] (a) $(CF_3SO_2)_2O$, Et_3N, CH_2Cl_2; (b) $ArB(OH)_2$ **19**, $Pd(PPh_3)_4$, CuI, Na_2CO_3, toluene-EtOH.

hydrogen bond with the carboxyl group of Thr179 of α-tubulin.[8] Moreover, compound **7** presents a methoxy group in front of the β-tubulin Cys241, which has largely been described to be involved in the interaction of colchicine analogues.[9,10] Curiously, compound **7** is more active than compound **8** that has a trimethoxyphenyl A-ring.

Thus, methoxy groups on A-ring seem to be important for the activity of 4-arylcoumarin compounds. The present report focuses on syntheses of a new series of analogues bearing the 3-hydroxy-4-methoxyphenyl moiety supposed to be crucial for activity and variously substituted on A-ring by methoxy groups (Scheme 2). The cytotoxic activities, the binding to tubulin, and microtubule inhibitory effects were reported for unsubstituted and mono-, di-, and trimethoxylated derivatives. It was important to state the P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) expression of cancer cell lines because some compounds need high concentrations to inhibit these pumps in the case of an association with anthracyclines. Finally, molecular calculations and modeling of the various compounds instructed the molecular mechanism of interaction.

RESULTS AND DISCUSSION

Chemistry. Several methods for preparing polysubstituted 4-phenylcoumarins involve direct arylation of activated coumarins using organometallic reagents. During the past five years, polymethoxylated neoflavones with potential cytotoxic activity were prepared in high yields under Suzuki reaction conditions using substituted coumarins and arylboronic acids.[7,11−16] Introduction of hydroxylated phenyl ring using protected arylboronic acids in the palladium-copper-catalyzed Suzuki coupling reaction requires a subsequent deprotection step to obtain the expected hydroxylated 4-arylcoumarins. As a selective deprotection is not reliable enough due to the presence of sensitive groups like benzopyrone, we focused our efforts on the possibility of preparing and using an unprotected arylboronic acid in the coupling reaction key step. 3-Benzyloxy-4-methoxyphenylboronic acid **18** was prepared by reported procedures involving the reaction of the appropriate aryllithium with triisopropylborate (Scheme 1).[15] Selective removal of the benzyl group was successfully performed by palladium-catalyzed hydrogenolysis, leading to the expected hydroxylated phenylboronic acid **19** in 87% yield.

Table 1. Structures and Yields of the 4-Arylcoumarins 1−8 of the Suzuki Coupling Reaction

	substituent			
product	R_1	R_2	R_3	yield (%)
unsubstituted				
1	H	H	H	81
monosubstituted				
2	OMe	H	H	65
3	H	OMe	H	97
4	H	H	OMe	98
disubstituted				
5	OMe	OMe	H	80
6	H	OMe	OMe	74
7	OMe	H	OMe	83
trisubstituted				
8	OMe	OMe	OMe	94

The activated 4-trifluorosulfonyloxycoumarins **10**−**17** were easily prepared in high yield by treatment of the appropriate 4-hydroxycoumarins with triflic anhydride as previously described.[7] Use of 3-hydroxy-4-methoxyphenylboronic acid **19** in coupling reaction led to the 4-(3-hydroxy-4-methoxyphenyl)coumarin derivatives **1**−**8** (Scheme 2) in good to high yield (65−98%, Table 1).

Biology. *Cytotoxicity.* Cytotoxicity of the synthesized compounds **1**−**8** was investigated in HBL100 human epithelial mammary cell line. A tetrazolium-based assay was used for the determination of the drug concentration required to inhibit cell growth by 50% after incubation in the culture medium for 72 h. The calculated IC_{50} values are collated in Table 2.

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Table 2. Cytotoxicity of CA-4 and 4-Arylcoumarins 1−8 towards HBL100 Cell Line

compd	CA-4	1	2	3	4	5	6	7	8
IC_{50} (nM)[a]	3 ± 0.3	84 ± 1	182 ± 10	39 ± 2	606 ± 22	439 ± 27	714 ± 29	88 ± 1	433 ± 29

[a] Drug concentration that inhibits the growth of HBL100 by 50% after incubation in liquid medium for 72 h. Data are the mean ± standard deviation (SD) of three experiments.

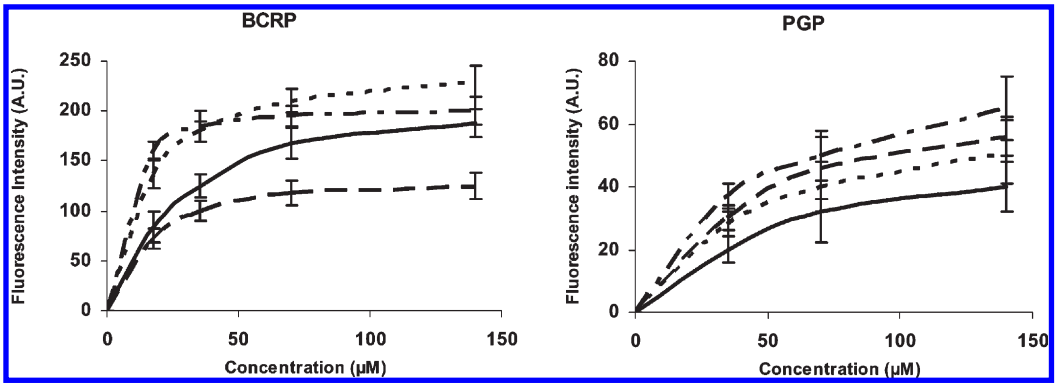

Figure 1. BCRP-overexpressing (R, resistant) HCT116 cells (left) and P-gp-overexpressing (R, resistant) K562 cells (right) were treated with variable doses of the four molecules **1** (—), **2** (---), **3** (— —), and **7**(— · —) (0−150 μM) and analyzed as described in the Experimental Section. Drug accumulation results are expressed in mean fluorescence intensity in arbitrary units (AU). Each drug concentration was tested in triplicate. Standard error (SE) of each point is <10%.

All compounds were found to exhibit potent cytotoxic activities in the nanomolar range. In particular, the IC_{50} value rate at 84, 182, 39, and 88 nM with **1**, **2**, **3**, and **7**, respectively, reflecting a highly pronounced antiproliferative activity. These results are in accordance with our previous reports dealing with the significance of 3′-OH and 4′-OMe groups on the 4-phenyl B-ring. Presence of the alkoxybenzene moiety is crucial to obtain relevant cytotoxic responses. In contrast, the phenyl A-ring can suffer several modifications and trimethoxy moiety is not as important as originally postulated. It appears that cytotoxicity might benefit from a less substituted A-ring. Furthermore an oxygenated group is not essential as unsubstituted compound **1** is one of the most cytotoxic compounds.

Interactions with P-gp and BCRP Pumps. On the basis of their cytotoxic effects, the most active compounds **1**−**3** and **7** were further studied in order to evaluate their activity as potential modulators of P-gp and BCRP ABC (ATP binding cassette) efflux pumps, which are involved in chemoresistance in various cancers.[17,18] We also tested if these compounds were substrates for P-gp and BCRP.

1. Evaluation of the Compounds on P-gp and BCRP Activity. In K562R and HCT116R cell lines, the four compounds restored the intracellular accumulation of daunorubicine (P-gp substrate) or mitoxantrone (BCRP substrate) with a concentration equivalent or higher to that of the BCRP and P-gp inhibitor cyclosporine A (Figure 1).

These results outlined in Table 3 show that compounds **2** and **7** were able to restore mitoxantrone accumulation (BCRP evaluation) at concentrations similar to that of cyclosporine A. Compound **7** was also the most efficient to reverse P-gp activity but at a concentration 3-fold higher than that of cyclosporine A. Compounds **1** and **3** displayed lower modulation activities on P-gp and BCRP compared to those of compound **7**. However, even if a modulation of P-gp and BCRP could be reached, the compounds concentrations needed were high. Therefore, in light of these results, only compound **7** is of interest in a clinical context of ABC pumps modulation as this molecule displayed the lowest concentration needed to reverse P-gp and BCRP.

Table 3. Modulation of P-gp and BCRP ABC Efflux Pumps

compd	1	2	3	7
reversion (μM)[a]				
P-gp	80	45	40	30
BCRP	20	10	40	10

[a] Compound concentration inducing the same reversion as the BCRP and P-gp inhibitor cyclosporine A (10 μM).

2. Evaluation of the Compounds as Potential Substrates of P-gp and/or BCRP. In the above experiments, when two molecules (mitoxantrone and one of the studied compounds) were administered together, a competitive mechanism for binding on P-gp or BCRP could occur. In the presence of one compound, the kinetics of binding with P-gp or BCRP could be quite different. This hypothesis was tested by treating HCT116S and R and K562S and R cells with 0.1 μM or 10 μM of compound **1**−**3** and **7** (Table 4) for 24 h, followed by cell cycle analysis. The effect of the four compounds on cell viability was also assessed by MTT after 48 h of treatment. A comparison of the IC_{50}s in the parental and resistant cell lines provided further evidence of the ability of these compounds to modulate the sensitivity of the cell lines in the presence or absence of P-gp or BCRP (Table 5).

At a concentration of 0.1 μM, compounds **1** and **3** were not able to induce a marked blockade in G2/M compared to the controls in K562R cells (bold data in Table 4). However, at 10 μM, the effects were the same in sensitive and in resistant cells. The IC_{50}s of the different compounds on the K562 cell line were quite similar (Table 5). The same kind of phenomenon was observed for compounds **1**, **2**, and **7**; at 0.1 μM there was no increase in G2/M compared to the control cells in HCT116R cell line. IC_{50}s were similar between HCT116S and R cells for compounds **1**, **2**, and **3**, whereas compound **7** exhibited a higher

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Table 4. Percentage of the Cells Blocked in G2/M by 4-Arylcoumarins 1−3 and 7[a]

	cells G2/M (%)								
		1 (μM)		**2** (μM)		**3** (μM)		**7** (μM)	
cell lines	control	0.1	10	0.1	10	0.1	10	0.1	10
K562S	23 ± 2	36 ± 5	43 ± 2	40 ± 3	47 ± 3	38 ± 5	40 ± 4	35 ± 2	43 ± 3
K562R	18 ± 2	**24** ± 3	40 ± 3	46 ± 1	42 ± 4	**26** ± 6	42 ± 3	35 ± 3	39 ± 3
HCT116S	32 ± 3	44 ± 2	48 ± 2	44 ± 3	60 ± 5	46 ± 4	56 ± 4	44 ± 2	50 ± 2
HCT116R	30 ± 4	**33** ± 3	49 ± 4	**27** ± 1	50 ± 6	39 ± 3	47 ± 4	**33** ± 3	43 ± 4

[a] Percentage of cells blocked in the G2/M phase of the cell cycle after 24 h incubation of the chemosensitive or chemoresistant cell lines with compounds **1**, **2**, **3**, and **7** at 0.1 mM or 10 mM. Data are the mean ± SD of three experiments. In bold, conditions for which no differences were observed between control and treated cells.

IC_{50} in the resistant form. This latter compound could thus be a weak substrate of BCRP.

The differences observed between cell cycle data and the IC_{50}s could be due to the fact that (i) IC_{50}s were evaluated after 48 h of treatment because there was only a moderate apoptosis after 24 h, (ii) the target of these derivatives could not only be tubulin but also signaling pathways as it was demonstrated for combretastatin A in the literature, (iii) the concentrations needed to block tubulin polymerization could be higher than those able to modify cell signaling and induce cell death. These results demonstrate that the four compounds are either not substrates (**1**, **2**, and **3**) or poor substrates (**7**) for P-gp or BCRP. As a blockade in the G2/M phase of the cell cycle was observed at various concentrations for all the cell lines tested in this study, irrespective of their tissue of origin (HBL100 is a mammary cell line, K562 is of hematopoietic origin and HCT116 is a colon cell line), this pointed toward a major common mechanism related to the cell cycle. The effects on tubulin assembly by the various compounds **1**−**8** were then investigated.

Effects on Tubulin Assembly. Figure 2 shows the effects of compound **1** on the fluorimetry time course of microtubule assembly from pure tubulin. For monitoring the microtubule formation, we used 4′,6-diamidino-2-phenylindole (DAPI) fluorescence. This well-known DNA binding agent binds to microtubules with a greater affinity than to tubulin dimer and its fluorescence was increased upon microtubule binding.[19,20] Therefore, DAPI is an interesting fluorophor to monitor the microtubule assembly because it does not affect the tubulin properties to form microtubule and its use is more sensitive than turbidimetric assay. By using this fluorimetric assay, a clear inhibition was noted, and the rate of assembly, as well as the final amount of microtubules, was lower in the presence of **1** than in the control experiment. A similar experiment (as a positive control inhibitor, line 6) performed with combretastatin A-4 at 3 μM showed a full inhibition of microtubule formation. The extent of inhibition by all compounds increased monotonically with the mole ratio of the total ligand to total tubulin in the solution (R). Table 6 indicates the half inhibitory molar ratio (ligand/tubulin) of microtubule formation in vitro. As an example for combretastatin A-4 (a substoichiometric inhibitor of microtubule formation), a mole ratio of 0.07 mol of combretastatin per mol of tubulin was necessary to halve microtubule formation. The most active compounds (substoichiometric inhibitors of the microtubule formation) were the A-ring-unsubstituted compound **1** and the monosubstituted compounds **2** and **3**. We noted that the third monosubstituted compound **4** had a lower activity, close to a stoichiometric mode of inhibition. For the disubstituted compounds **5** and **6**, it was necessary to add an excess of ligand to inhibit microtubule formation. In contrast, the disubstituted compound **7** was found to be a substoichiometric inhibitor of microtubule formation. Finally, the trisubstituted compound **8** had a weaker activity against microtubule formation.

Table 5. Cytotoxicity of 4-Arylcoumarins 1−3 and 7 towards K562 and HCT116 Cell Lines

	IC_{50} (nM)[a]			
cell line	**1**	**2**	**3**	**7**
K562**S**	96 ± 10	86 ± 7	88 ± 8	100 ± 10
K562**R**	92 ± 4	86 ± 11	78 ± 12	88 ± 9
HCT116**S**	100 ± 11	190 ± 13	150 ± 9	210 ± 8
HCT116**R**	120 ± 10	200 ± 12	140 ± 9	310 ± 7

[a] Drug concentration that inhibits the cell growth by 50% after incubation in liquid medium for 48 h. Data are the mean ± SD of three experiments.

Interaction of Compounds with Tubulin: Binding Parameters. The uncorrected intrinsic fluorescence emission spectra of tubulin were examined by exciting the tryptophan residues at 295 nm. A decrease in fluorescence emission intensity at 340 nm was observed with increasing concentrations of compound **1** (Figure 3). This quenching of fluorescence was used to perform binding titration experiments. Quenching of tubulin fluorescence was observed for all compounds and suggests that compounds bind to tubulin close to fluorophore, i.e., tryptophan and tyrosine residues. The inset of Figure 3 shows the titration curve for the association of compound **1** to tubulin. The apparent affinity stoichiometric binding constant was calculated. We found a K_a value of $(1.41 \pm 0.52) \times 10^6\ M^{-1}$ for compound **1**. Similar experiments and assumptions were used to determine the binding affinity of the other analogues **2**−**8** (Table 6).

The unsubstituted compound **1** and the monosubstituted compound **2** had the highest apparent affinity binding constant, in the same range as that of combretastatin A-4, i.e., about $1 \times 10^6\ M^{-1}$. These latter molecules were the most efficient inhibitors of microtubule formation in vitro. Then **3**, **4**, and **7** had a less pronounced interaction with protein but still have a substoichiometric mode of inhibition. Finally, less potent in tubulin assembly inhibition, with a stoichiometric mode of inhibition, **5**, **6**, and **8** have the lowest apparent affinity binding constants. Thus, the simplest correlation between affinity and inhibitory capacity seems easily feasible.

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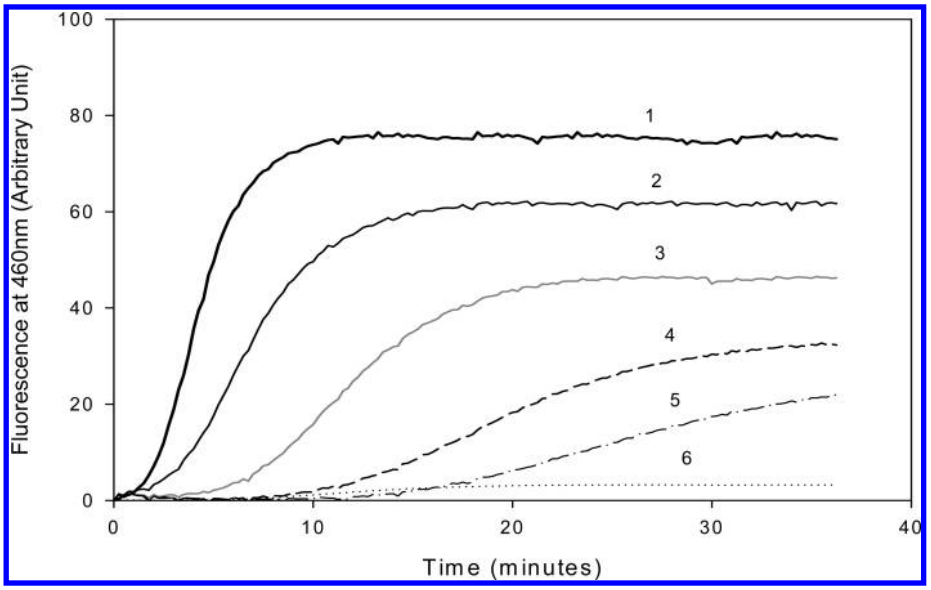

Figure 2. Effect of **1** on in vitro microtubule assembly. Panel shows tubulin alone (line 1) at 15 μM and with various concentrations of compound **1** at 1.5 μM (line 2), 2.7 μM (line 3), 4.4 μM (line 4), 5.5 μM (line 5). Line 6 is a positive control in the presence of combretastatin A-4 at 3 μM.

Table 6. Inhibitory Effects on Microtubule Assembly at 37 °C and Binding Parameters of Combretastatin A-4 and Compounds 1−8 to Tubulin in PG Buffer at 25 °C[a]

compd	half inhibitory molar ratio (ligand/tubulin) of microtubule formation in vitro	equilibrium binding constant to tubulin K_a [M^{-1}]
combretastatin A-4	0.07	$(3.35 \pm 1.46) \times 10^{6}$[b]
1	0.18	$(1.41 \pm 0.52) \times 10^{6}$
2	0.16	$(1.64 \pm 0.80) \times 10^{6}$
3	0.23	$(7.40 \pm 1.10) \times 10^{5}$
4	0.91	$(4.55 \pm 1.23) \times 10^{5}$
5	2.31	$(1.34 \pm 0.47) \times 10^{5}$
6	2.68	$(2.47 \pm 0.70) \times 10^{5}$
7	0.29	$(3.75 \pm 1.23) \times 10^{5}$[b]
8	1.50	$(6.72 \pm 0.88) \times 10^{4}$[b]

[a] Values are the mean of three experiments. K_a values are given ± SD of the mean. [b] Determined in the author's laboratory in a previous work.[7,8]

Molecular Structure and Biological Activity. The molecular structure of 5,7-dimethoxycoumarin derivative **7**, previously studied in our group,[7,8,16] was determined by single-crystal X-ray crystallography and compared to the molecular structure of combretastatin A-4.[21] The ORTEP diagram of compound **7** is presented in Figure 4, while selected parameters are listed in Table 7. The tridimensional structure revealed a molecular conformation in which the two aromatic rings, A and B, are twisted and form the same conformation of the diaryl system than combretastatin A-4, with a very similar torsion angle (48° compared to 53°, respectively). These hydrophobic planar groups are crucial for antiproliferative activity and serve as the rigid moiety of the molecular scaffold that should satisfy the overall geometric and steric requirements of binding with tubulin.

To explain the relative biological activity of the different compounds (combretastatin A-4, **1**−**8**) theoretical calculations were performed at the semiempirical level using the Ampac 6.55 package[22] and at the DFT level using the Gaussian G03 software.[23] All the calculations were carried out on a Silicon Graphics Origin 2000 R12000 workstation. Because the molecules have numerous possible conformations, we started with the extracted X-ray structure of combretastatin A-4 and compound **7**. After this step, the built geometries were optimized without constraints at the DFT B3LYP/6−31G(d) level of theory. Superimpositions of B-ring of optimized DFT compounds **1**−**8** are shown in Figure 5, while selected parameters are listed in Table 8.

The superimposition of compounds **1**−**8** based on B-ring showed an extensive overlapping of the chromenone core. This result supports the hypothesis that the spatial arrangement of the two aromatic rings (A) and (B) plays an essential role in the activity and binding of compounds that bind to the colchicine domain (main binding site and additional pockets) on α- and β-tubulin. The 3′-OH and 4′-OMe substituents on the 4-phenyl B-ring are crucial for activity and were identified as pharmacophoric groups. The slight difference of biological responses observed can be discussed in terms of the substitution pattern and the number of methoxy groups on the phenyl A-ring. Vicinal dimethoxy-substituted derivatives (**5**, **6**, and **8**) were 8−15-fold less active than unsubstituted compound **1**. Therefore, it appears clearly that the presence of a constraint methoxy group out of the A-ring plane is a detrimental parameter for the activity against tubulin assembly and binding. This steric hindrance factor seems to play a pivotal role in accommodating the A-ring inside the active binding pocket on tubulin. Moreover, the potent inhibitory effect and large binding affinity constant for compound **1** indicates that 4-arylcoumarin compounds bind to colchicine domain inside additional pockets buried deeper in the tubulin heterodimer. This situation is similar to that of the tubulin vinca alkaloïds domain that is described as consisting of a core targeted by all ligands that interact with vinblastine for tubulin binding and of ligand-dependent extensions.[24]

■ CONCLUSION

It has been long thought that the presence of the trimethoxybenzene moiety was crucial to obtain relevant cytotoxic and antitubulin

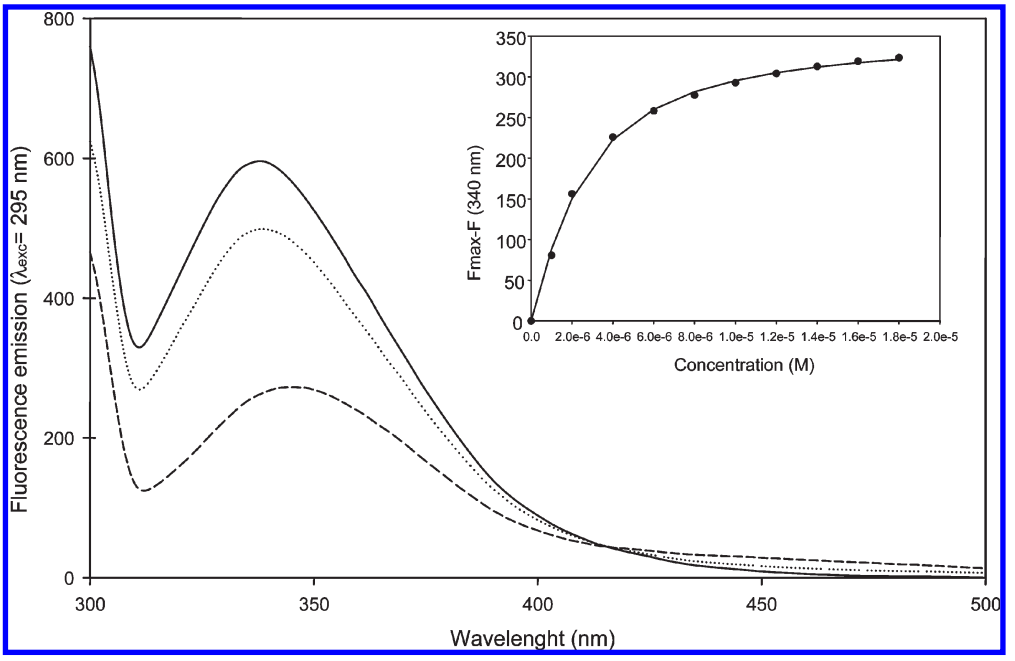

Figure 3. Tryptophan fluorescence modifications of tubulin upon binding of compound **1** at 25 °C in PG buffer. The excitation wavelength was 295 nm. Fluorescence emission of 3.2 μM tubulin (—), with 1 μM (· · ·), and 12 μM (---) compound **1**. Inset: quenching fluorescence titration curve of 3.2 μM tubulin with various compound **1** concentrations. This curve was analyzed as described in materials and methods, and for this experiment, an affinity constant of $(1.1 \pm 0.2) \times 10^6$ M^{-1} was obtained.

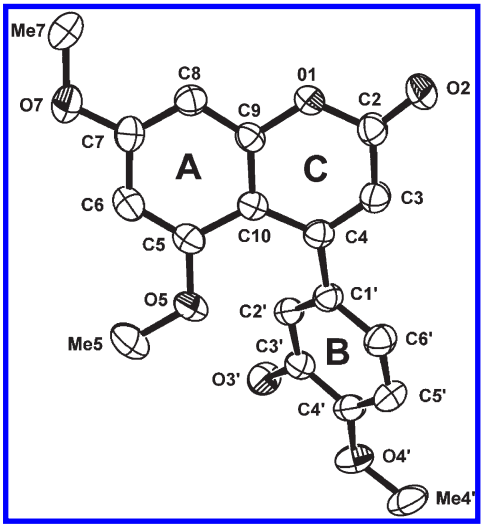

Figure 4. ORTEP drawing of compound **7** (all hydrogen atoms are omitted for clarity).

Table 7. X-ray Crystallographic Data of Compound 7

torsion angle	deg	distance	Å
ring (A)−ring (B)	48.3	centroid (A)-O4′	7.721[a]
ring (A)−5-OMe	5.8	centroid (A)-Me4′	8.786[b]
ring (A)−7-OMe	−11.6	centroid (A)-O3′	6.250[c]

[a] CA-4: 7.377 Å. [b] 8.586 Å. [c] 5.629 Å.

responses. The modifications of the trimethoxyphenyl A-ring demonstrated that the methoxy groups are not essential for the interaction and activity of the 4-arylcoumarin compounds.

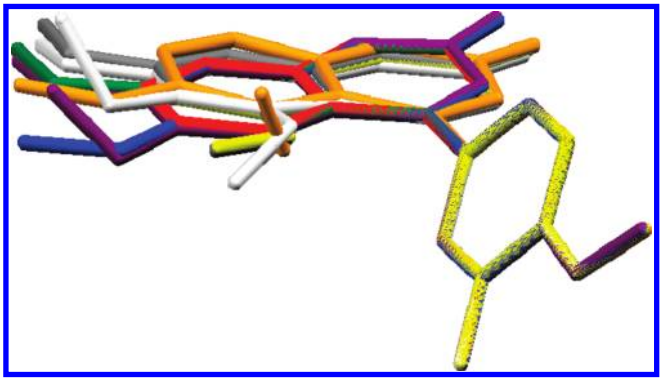

Figure 5. Superimposition of the B-ring of compounds **1** (red), **2** (yellow), **3** (blue), **4** (green), **5** (orange), **6** (purple), **7** (gray), and **8** (white).

Unsubstituted compound **1** and monosubstituted compound **2** showed a substoichiometric antitubulin activity comparable to that of combretastatin A-4 with a highly similar apparent affinity binding constant. The slight loss of potency for the other synthesized compounds was correlated with a decrease in the values of their affinity binding constants. The interaction with protein surface appeared down regulated by the presence of a methoxy group directed up to the molecular plane of ligand. Previous to a use in an in vivo model the P-gp and BCRP expression of cancer cells was stated. In the case of a single use in a chemotherapeutic regimen, a concentration of 10 μM of compounds seems to be needed to be insensitive to the efflux activity. In addition to that sum of results, this work provide a consistent experimental basis to guide the design of new potent antitubulin agents that target the whole colchicine binding domain.

Table 8. DFT Calculation Data of Combretastatin A-4 and Compounds 1−8

compd	C A-4	1	2	3	4	5	6	7	8
				Torsion Angle (deg)					
ring (A)−ring (B)	53.0	57.9	63.2	57.4	57.7	63.5	56.5	63.2	64.5
Me7O7C7C8	172.2				0.4		3.3	0.5	3.7
Me6O6C6C7	93.4			−0.1		4.0	**−59.5**		**−66.9**
Me5O5C5C6	8.6		0.5			**−63.2**		0.7	36.4
				Distance (Å)					
centroid (A)−O4′	7.377	7.600	7.818	7.610	7.608	7.848	7.600	7.817	7.790
centroid (A)−Me4′	8.587	8.662	8.858	8.673	8.671	8.869	8.665	8.856	8.833
centroid (A)−O3′	5.629	6.301	6.557	6.304	6.305	6.639	6.286	6.564	6.522

EXPERIMENTAL SECTION

Chemistry. Melting points were taken on a Büchi capillary apparatus and are uncorrected. NMR spectra were obtained on a Bruker AC 300 spectrometer. Chemical shifts (δ) are reported in ppm for a solution of the compound in $CDCl_3$ with internal reference Me_4Si and J values in hertz. Combustion analyses were performed at the Laboratoire de Microanalyse of the Centre National de la Recherche Scientifique, Vernaison, and all final compounds have a purity of >95%. Separation by column chromatography was performed using Merck Kieselgel 60 (70−230 mesh). Ether refers to diethyl ether and petroleum spirit refers to the fraction with distillation range 40−65 °C. All solvents were purified by standard techniques. 3-Benzyloxy-4-methoxyphenylboronic acid **18** was prepared by reported procedures involving reaction of the appropriate aryllithium with triisopropylborate.[15] 4-Trifluoromethylsulfonyloxychromen-2-one **10**,[25] 5-methoxy-4-trifluoromethylsulfonyloxychromen-2-one **11**,[25] 6-methoxy-4-trifluoromethylsulfonyloxychromen-2-one **12**,[26] 7-methoxy-4-trifluoromethylsulfonyloxychromen-2-one **13**,[15,27] 5,7-dimethoxy-4-trifluoromethylsulfonyloxychromen-2-one **16**,[25] and 5,6,7-trimethoxy-4-trifluoromethylsulfonyloxychromen-2-one **17**[7] were prepared as previously reported.

3-Hydroxy-4-methoxyphenylboronic Acid 19. A mixture of 3-benzyloxy-4-methoxyphenylboronic acid **18** (5.16, 20 mmol) and 10% palladium on graphite (2 w%) in dry tetrahydrofuran (40 mL) was stirred under hydrogene atmosphere for 24 h. The mixture was diluted with acetone and filtered through celite, and the filtrate was concentrated under reduced pressure. The solid residue was washed with 50% ether−pentane and collected to afford 3-hydroxy-4-methoxyphenylboronic acid **19** as white powder (2.86 g, 85%), mp 221 °C. ^{1}H NMR (acetone-d_6, 300 MHz): δ_H 3.87 (3H, s), 6.91 (2H, s), 6.94 (1H, d, J 8.3), 7.35 (1H, s), 7.88 (1H, d, J 1.6), 7.38 (1H, dd, J 8.3).

Preparation of the 4-Trifluoromethylsulfonyloxychromen-2-ones: General Procedure. Trifluoromethanesulfonic anhydride (0.66 mL, 1.3 equiv) was added dropwise over 10 min to a mixture of the appropriate 4-hydroxycoumarin (3 mmol) and triethylamine (0.55 mL, 1.3 equiv) in dry dichloromethane (20 mL) at 0 °C. Then the mixture was stirred for 1 h at 0 °C. After dilution with 50% ether−petroleum spirit (50 mL) and filtration through a short pad of silica, the solvent was distilled off under reduced pressure to a small volume (10 mL) and the residue kept at −15 °C overnight. The precipitate was collected and washed with petroleum spirit to afford the expected 4-trifluoromethylsulfonyloxychromen-2-one.

5,6-Dimethoxy-4-trifluoromethylsulfonyloxychromen-2-one ***14***. Light-yellow needles, 97%, mp 95−98 °C; δ_H 3.91 (3H, s), 3.97 (3H, s), 6.31 (1H, s), 7.12 (1H, d, J 8.6), 7.24 (1H, d, J 8.6); δ_C 56.6, 61.6, 108.4, 109.2, 112.2, 118.3, 118.5, 144.7, 147.5, 149.6, 157.1, 159.5.

6,7-Dimethoxy-4-trifluoromethanesulfonyloxycoumarin ***15***. White needles, 73%, mp 100 °C; ^{1}H NMR (acetone-d_6, 300 MHz): δ^H 3.95 (3H, s), 4.01 (3H, s), 6.35 (1H, s), 6.91 (1H, s), 6.99 (1H, s); δ_C 56.4, 56.7, 100.3, 102.2, 102.9, 106.1, 118.5, 147.2, 150.0, 154.9, 157.4, 160.4.

Coupling of 4-Trifluoromethylsulfonyloxycoumarins with Arylboronic Acids: General Procedure. A mixture of 4-trifluoromethylsulfonyloxycoumarin (1 mmol), tetrakis(triphenylphosphine)palladium (0) (46 mg, 4 mol%), copper(I) iodide (210 mg, 1.1 equiv), sodium carbonate (742 mg, 7 equiv), and arylboronic acid (252 mg, 1.5 equiv) in dry benzene (10 mL) and absolute ethanol (3 mL) was refluxed overnight. Then the reaction mixture was diluted with chloroform (40 mL) and filtered through celite. The filtrate was washed with a saturated aqueous solution of sodium bicarbonate (3 × 20 mL), and the combined aqueous layers were extracted with chloroform (3 × 20 mL). The organic phases were combined, washed with brine, and dried over Na_2SO_4, and the solvent was distilled under reduced pressure. The residue was crystallized from ether or purified by column chromatography to afford the 4-arylcoumarin derivative.

4-(3′-Hydroxy-4′-methoxyphenyl)coumarin ***1***. Purified by column chromatography, eluent Et_2O−pentane (7:3), as white needles, 81%, mp 172 °C; δ_H 3.99 (3H, s), 5.78 (1H, s), 6.36 (1H, s), 6.97 (1H, dd, J 8.1 and 1.3), 7.00 (1H, d, J 8.1), 7.06 (1H, d, J 1.3), 7.23 (1H, t, J 7.9), 7.40 (1H,d, J 7.9), 7.54 (1H, td, J 7.9 and 1.5), 7.60 (1H, dd, J 7.9 and 1.5); δ_C 56.1, 110.9, 114.6, 114.9, 117.3, 119.0, 120.7, 124.2, 127.2, 128.3, 131.8, 145.9, 147.9, 154.2, 155.4, 161.1. Anal. ($C_{16}H_{12}O_4$) C, H.

4-(3′-Hydroxy-4′-methoxyphenyl)-5-methoxycoumarin ***2***. Crystallized from Et_2O as light-brown crystals, 65%, mp 180 °C; δ_H 3.53 (3H, s), 3.94 (3H, s), 6.15 (1H, s), 6.67 (1H, dd, J 8.3 and 0.7), 6.79 (1H, dd, J 8.1 and 1.9), 6.86 (1H, d, J 8.1), 6.87 (1H, d, J 1.9), 7.00 (1H, dd, J 8.3 and 0.7) and 7.44 (1H, t, J 8.3); δ_C 55.6, 55.9, 106.7, 109.2, 109.6, 109.7, 114.1, 115.6, 118.9, 132.2, 132.7, 144.7, 146.7, 155.1, 155.3, 157.3, 160.6. Anal. ($C_{17}H_{14}O_5$) C, H.

4-(3′-Hydroxy-4′-methoxyphenyl)-6-methoxycoumarin ***3***. Crystallized from Et_2O as light-brown crystals, 97%, mp 167 °C; δ_H 3.76 (3H, s), 3.99 (3H, s), 6.34 (1H, s), 6.96 (1H, dd, J 8.2 et 1.7), 6.99 (1H, d, J 8.2), 7.04 (1H, d, J 2.8), 7.06 (1H, d, J 1.7), 7.12 (1H, dd, J 9.0 and 2.8), 7.33 (1H, d, J 9.0); δ_C 55.9, 56.1, 110.0, 110.9, 114.9, 115.1, 118.2, 119.0, 119.5, 120.5, 128.4, 146.2, 148.0, 148.6, 155.2, 155.9, 161.3. Anal. ($C_{17}H_{14}O_5$) C, H.

4-(3′-Hydroxy-4′-methoxyphenyl)-7-methoxycoumarin ***4***. Crystallized from Et_2O as light-brown crystals, 98%, mp 173 °C (lit.[28] 169−171 °C); δ_H 3.89 (3H, s), 3.98 (3H, s), 6.19 (1H, s), 6.79 (1H, dd, J 8.8 and 2.7), 6.88 (1H, d, J 2.7), 6.95 (1H, dd, J 8.1 and 1.5), 6.98 (1H, d, J 8.1), 7.04 (1H, d, J 1.5), 7.49 (1H, d, J 8.8); δ_C 55.0, 55.2, 100.3, 110.3, 110.8, 111.4, 111.8, 114.6, 119.5, 127.6, 127.7, 146.0, 148.0, 155.0, 155.4, 160.3, 162.3. Anal. ($C_{17}H_{14}O_5$) C, H.

4-(3′-Hydroxy-4′-methoxyphenyl)-5,6-dimethoxycoumarin ***5***. Crystallized from Et_2O as light-brown crystals, 80%, mp 165−166 °C; δ_H 3.24 (3H, s), 3.85 (3H, s), 3.93 (3H, s), 6.20 (1H, s), 6.84 (1H, dd, J 8.3 and 1.5), 6.86 (1H, d, J 8.3), 6.96 (1H, d, J 1.5), 7.11 (1H, d, J 9.2), 7.16 (1H, d, J 9.2); δ_C 55.9, 56.5, 60.9, 109.7, 111.9, 114.0, 114.3, 116.3,

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117.2, 119.4, 131. 7, 144.7, 146.3, 148.1, 149.6, 154.6, 160.6. Anal. ($C_{18}H_{16}O_6$) C, H.

4-(3′-Hydroxy-4′-methoxyphenyl)-6,7-dimethoxycoumarin ***6***. Crystallized from Et_2O as light-yellow crystals, 66%, mp 208 °C; δ_H 3.79 (3H, s), 3.97 (3H, s), 3.99 (3H, s), 5.55 (1H, s), 6.22 (1H, s), 6.91 (1H, s), 6.97−6.99 (3H, m), 7.06 (1H, s); δ_C 56.1, 56.4, 56.5, 100.3, 107.6, 110.9, 111.6, 112.0, 114.7, 120.5, 129.0, 146.0, 146.1, 147.8, 150.2, 152.9, 155.4, 161.7. ($C_{18}H_{16}O_6$) C, H.

4-(3′-Hydroxy-4′-methoxyphenyl)-5,7-dimethoxycoumarin ***7***. Purified by column chromatography, eluent Et_2O, as white needles, 83%, mp 152 °C (lit.[7] 152 °C); δ_H 3.50 (3H, s), 3.86 (3H, s), 3.94 (3H, s), 5.82 (1H, s), 5.99 (1H, s), 6.24 (1H, d, *J* 2.3), 6.50 (1H, d, *J* 2.3), 6.78 (1H, dd, *J* 8.1 and 1.9), 6.85 (1H, d, *J* 8.1), 6.86 (1H, d, *J* 1.9). ($C_{18}H_{16}O_6$) C, H.

4-(3′-Hydroxy-4′-methoxyphenyl)-5,6,7-trimethoxycoumarin ***8***. Purified by column chromatography, eluent Et_2O, as white fine needles, 94%, mp 157 °C (lit.[7] 157 °C); δ_H 3.36 (3H, s), 3.80 (3H, s), 3.94 (3H, s), 3.95 (3H, s), 6.06 (1H, s), 6.71 (1H, s), 6.83 (1H, dd, *J* 8.2 and 2.1), 6.99 (1H, d, *J* 8.2), 6.92 (1H, d, *J* 2.1); δ_C 56.0, 56.3, 61.1, 61.2, 96.3, 107.4, 109.8, 114.1, 114.2, 119.3, 132.3, 139.6, 144.7, 146.7, 151.3, 151.8, 155.2, 156.8, 160.8. Anal. ($C_{19}H_{18}O_7$) C, H.

Biology. *Cytotoxicity Assays.* Cells were seeded in 96-well plates to be treated during 72 h. Growth inhibition of HBL100 cell line was studied after a 72 h treatment with different compounds by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) cell proliferation assay, and absorbance was measured at 550 nm with a Dynatech MR 7-000 plate reader. Three independent experiments were performed, and the IC_{50} values (i.e., concentration half inhibiting cell proliferation) were graphically determined.

Evaluation on P-gp and BCRP Activity. Accumulation studies of mitoxantrone (BCRP substrate) and daunorubicin (P-gp substrate) were performed using wild type (sensitive) HCT116S cell line, which does not express BCRP, and the BCRP-transfected resistant form HCT116R, and on wild type (sensitive) K562S and the P-gp expressing (resistant) K562R cell lines.[29] Cells grown in RPMI 1640 medium with 10% FCS were trypsinized (HCT116), washed twice, then resuspended in RPMI 1640 medium with 10% FCS to obtain a density of 10^6 cells/mL. Various concentrations (0−150 μM) of 4-arylcoumarin compounds or the BCRP and P-gp inhibitor cyclosporine A (10 μM final concentration) were added to 1 mL of cells and incubated for 15 min at 37 °C, followed by the addition of mitoxantrone (final concentration 3 μM) or daunorubicin (final concentration 1 μM). After 30 min of incubation at 37 °C, 4 mL of ice-cold PBS were added to stop drug accumulation and fluorescence was quantified by flow-cytometry and analyzed using CELLQUEST PRO software (Becton Dickinson Sciences).

Evaluation as Potential Substrates of P-gp and/or BCRP. Cell Cycle Analysis. HCT116S and R, K562S and R exponentially growing cells were incubated for 24 h in the presence of DMSO (vehicle/0.1%) or 4-arylcoumarin compounds at 0.1 and 10 μM. Cell DNA content was analyzed using the CycleTest PLUS/DNA reagent kit (BD Sciences, San Jose, CA). Data were collected and analyzed on a FACSCalibur flow-cytometer (Becton Dickinson, La Jolla, CA) using CELLQUEST PRO software (BD Sciences, Le Pont de Claix France).

Cytotoxicity Assays. HCT116S and R, K562S and R were cultured in 96-well plates for 48H. The number of viable cells was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described by the manufacturer (Sigma Aldrich, l'Isle d'Abeau, France). Three independent experiments were performed and the IC_{50} values (concentration half inhibiting cell proliferation) were graphically determined.

Tubulin Purification. Tubulin was extracted from lamb brains by ammonium sulfate fractionation and ion exchange chromatography and stored in liquid nitrogen.[8] Before use, aliquots of protein were chromatographed in drained spin columns (1 cm × 5 cm) of Sephadex G25, equilibrated with PG buffer (20 mM sodium phosphate, 0.1 mM GTP, pH 7), followed by passage through a gravity column of Sephadex G25 (1 cm × 10 cm) and equilibrated with the same buffer. Protein concentration was measured spectrophotometrically with a Perkin-Elmer spectrophotometer Lambda 800 at 275 nm with an extinction coefficient of 1.09 L g^{-1} cm^{-1} in guanidine hydrochloride or 1.07 L g^{-1} cm^{-1} in 0.5% SDS in neutral aqueous buffer.

Tubulin Assembly. Microtubule assembly was monitored on a Fluoroscan Ascent FL spectrofluorometer (Labsystems) using a 96-well plate.[30] The excitation wavelength was set at 355 nm, and the emission wavelength was set at 460 nm. Experiments were carried out at 37 °C and performed with 7.5 μM Dapi, 15 μM tubulin in 20 mM sodium phosphate buffer, 1 mM ethylene glycol tetraacetic acid (EGTA), 10 mM $MgCl_2$, 10^{-4} M GTP, and 3.4 M glycerol, pH 6.5. Under these conditions, the Dapi fluorescence enhancement is directly proportional to the concentration of polymerized tubulin and was monitored as a function of time.[31] Me_2SO concentration was maintained below 4% in all samples and controls. Experiments were done in triplicate

Binding Measurement by Fluorescence Titration. Fluorescence measurements were performed with a Perkin-Elmer luminescence spectrometer 50 with slit widths of 10/10 nm. Uncorrected fluorescence spectra were obtained in PG buffer, pH 7, by using 0.2 cm (excitation direction) × 1 cm cells (Hellma) thermostatted at 25 °C by circulating water from an external water bath. Determination of the binding parameters was done by measuring quenching of the intrinsic protein fluorescence signal by ligands. Tubulin (1−4 μM) was titrated with various concentrations of analogues. Fluorescence measurements were performed with an excitation wavelength of 295 nm in order to specifically excite the tubulin tryptophanyl residues.

Fluorescence Binding Titration for Compounds. Fluorescence emission was recorded at 340 nm. The inner filter effects were corrected according to the procedure as follows:[32]

$$F_{corr} = F_{obs} \exp\{(A_{exc} + A_{em})/2\}$$

F_{obs} and F_{corr} are the observed and corrected fluorescence values at the emission wavelengths. A_{exc} and A_{em} are the absorptions at the excitation and emission wavelengths, respectively, calculated with $A_x = \varepsilon_x l_x C$, in which x is the excitation or emission direction, ε is the extinction coefficient, l is the path length of the cell in the excitation and emission directions, and C is the ligand concentration. The corrected quenching fluorescence titration curves were inverted and fitted to the saturation curve equation by means of nonlinear least-squares regression analysis.

$$F_{corr} = \frac{F_{max}[L_f]}{K_d + [L_f]}$$

F_{max} is the plateau fluorescence value. Concentrations (bound ligand $[L_b]$ and free ligand $[L_f]$) and K_d the stoichiometric dissociation binding constant were determined with the following equation:

$$[L_b] = \frac{1}{2}\{([L_0] + [P_0] + K_d) - (([L_0] + [P_0] + K_d)^2 - 4[P_0][L_0])^{1/2}\}$$

where $[L_0]$ and $[P_0]$ are the total ligand and protein concentrations, respectively. The algorithm starts with an arbitrary opening K_d value. With this value, $[L_b]$ and $[L_f]$ were calculated and then the nonlinear least-squares regression analyses were executed. The initial set is corrected in the next step by a Newton−Gauss procedure. This iterative procedure is continued until the minimum sum of squared deviations between experimental and calculated values of F_{corr} is obtained.

The concentrations of compounds were measured spectrophotometrically. The extinction coefficients, ε, were determined by dissolving dry powder of the compounds in Me_2SO (dimethylsulfoxide) and diluting in 20 mM NaPi buffer, pH 7, then taking the UV visible spectra. Three independent determinations gave the values displayed in Table 9.

Table 9. Extinction Coefficients of 4-Arylcoumarins 1−6

compd			
1	$\varepsilon_{320\ nm} = 12400 \pm 640\ M^{-1}\ cm^{-1}$	$\varepsilon_{295\ nm} = 11190 \pm 510\ M^{-1}\ cm^{-1}$	$\varepsilon_{340\ nm} = 7300 \pm 310\ M^{-1}\ cm^{-1}$
2	$\varepsilon_{330\ nm} = 12630 \pm 800\ M^{-1}\ cm^{-1}$	$\varepsilon_{295\ nm} = 10306 \pm 550\ M^{-1}\ cm^{-1}$	$\varepsilon_{340\ nm} = 7794 \pm 420\ M^{-1}\ cm^{-1}$
3	$\varepsilon_{309\ nm} = 14500 \pm 950\ M^{-1}\ cm^{-1}$	$\varepsilon_{295\ nm} = 12614 \pm 700\ M^{-1}\ cm^{-1}$	$\varepsilon_{340\ nm} = 7930 \pm 255\ M^{-1}\ cm^{-1}$
4	$\varepsilon_{320\ nm} = 13380 \pm 750\ M^{-1}\ cm^{-1}$	$\varepsilon_{295\ nm} = 11195 \pm 950\ M^{-1}\ cm^{-1}$	$\varepsilon_{340\ nm} = 7008 \pm 350\ M^{-1}\ cm^{-1}$
5	$\varepsilon_{295\ nm} = 14093 \pm 650\ M^{-1}\ cm^{-1}$	$\varepsilon_{295\ nm} = 14093 \pm 500\ M^{-1}\ cm^{-1}$	$\varepsilon_{340\ nm} = 6212 \pm 290\ M^{-1}\ cm^{-1}$
6	$\varepsilon_{340\ nm} = 11140 \pm 850\ M^{-1}\ cm^{-1}$	$\varepsilon_{295\ nm} = 6911 \pm 360\ M^{-1}\ cm^{-1}$	$\varepsilon_{340\ nm} = 11140 \pm 645\ M^{-1}\ cm^{-1}$

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ABBREVIATIONS

P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; CA-4, combretastatin A4; CA-4P, combretastatin A-4 phosphate; ITC, isothermal titration calorimetry; THF, tetrahydrofuran; SE, standard error; SD, standard deviation; AU, arbitrary units; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4′,6-diamidino-2-phenylindole; EGTA, ethylene glycol tetraacetic acid

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