9-Benzylidene-naphtho[2,3-*b*]thiophen-4-ones as Novel Antimicrotubule Agents—Synthesis, Antiproliferative Activity, and Inhibition of Tubulin Polymerization

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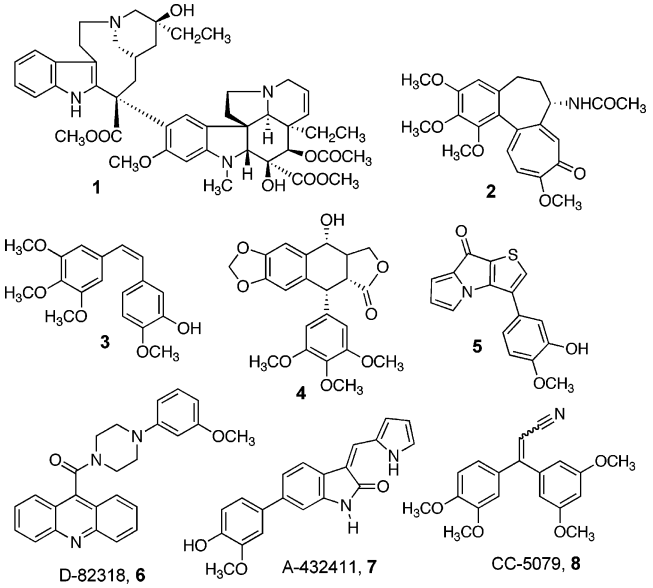
A novel series of 9-benzylidene-naphtho[2,3-*b*]thiophen-4-ones and structurally related compounds were synthesized and evaluated for their ability to inhibit tubulin polymerization. The 4-hydroxy-3,5-dimethoxy-benzylidene analogue **15d** was identified as a potent cytotoxic agent in an assay based on K562 leukemia cells. Antiproliferative activity of **15d** and the 2,4-dimethoxy-3-hydroxy-benzylidene analogue **15e** was additionally evaluated against a panel of 12 tumor cell lines, including multidrug resistant phenotypes. All resistant cell lines were sensitive to these compounds. Concentration-dependent flow cytometric studies showed that K562 cells as well as KB/HeLa cells treated by **15d** were arrested in the G2/M phases of the cell cycle. Moreover, four compounds strongly inhibited tubulin polymerization with activities higher or comparable to those of the reference compounds. In competition experiments, the most active compounds strongly displaced radiolabeled colchicine from its binding site in the tubulin, showing IC_{50} values virtually 3- to 4-fold lower than that of colchicine.

Introduction

Drugs that target microtubules were first discovered decades ago, and various strategies to affect the mitotic spindle have meanwhile been studied. Nevertheless, microtubules remain one of the main targets for the development of potential new chemotherapeutic agents.[1,2] Microtubules are hollow tubes found in all eukaryotic cell types. Microtubules are essential cytoskeletal polymers, and they affect cell shape, segregation of chromosomes during mitosis, organization of intracellular structure, and transport. Tubulin together with its isotypes, the building block of microtubules, exists as a heterodimer of α- and β-tubulin. Numerous antimitotic drugs displaying a wide structural diversity have been identified to interfere with the tubulin system.[3,4] Almost all of them interact with the α/β-tubulin dimer, rather than microtubule associated proteins (MAPs[a]) or other proteins involved in microtubule functions. The vinca alkaloids vinblastine (**1**, Chart 1**)** and vincristine, as well as the taxanes, such as taxol (INN: paclitaxel) and taxotere (INN: docetaxel) are widely used clinically as important chemotherapeutic agents for the treatment of many malignancies. It was mainly the discovery of these compounds that stimulated intense research aimed at additional microtubule-targeting drugs. Colchicine (**2**) has limited medicinal utility due to its narrow therapeutic window, but played an important role in elucidation of the properties and functions of tubulin and microtubules. Many natural products, such as combretastatin A-4[5] (**3**), podophyllotoxin (**4**), or the epothilones,[6] as well as some more synthetic compounds including thienopyrrolizinone[7] (**5**), acridinyl-9-carboxamide D-82318[8] (**6**), indolinone A-432411[9] (**7**), or propenenitrile CC-5079[10] (**8**; Chart 1), to cite just a few, are known to mediate cytotoxic activities through a binding interaction with tubulin. Within the past few years, especially small molecular colchicine-site binders were vigorously pursued as new antimitotic agents.[4]

Chart 1. Examples of Tubulin Interacting Agents



We previously reported the potent in vitro antitumor activity of 10-[(3-hydroxy-4-methoxy-benzylidene)]-9(10*H*)-anthracenone (**9**, Chart 2**)**, a potent colchicine-site binder with an IC_{50} of 0.67 μM against tubulin polymerization and 20 nM against K562 cell growth.[11] Structure−activity relationship studies were started to develop more potent anticancer agents and to understand the minimum structural requirements of this class of compounds to exhibit potent and selective activity against

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[a] Abbreviations: INN, international nonproprietary name; Kip, kinase inhibitor protein; MAPs, microtubule-associated proteins; MDR, multi-drug resistant; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; T/C, treated/untreated; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-*2H*-tetrazolium-5-carboxanilide.

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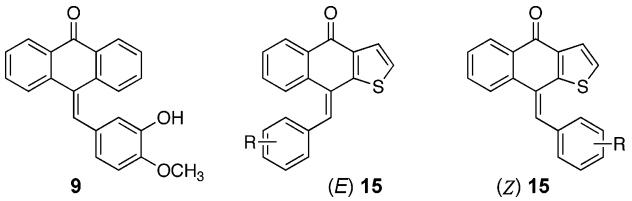
Chart 2. Structure of **9** and Geometric Isomers of **15**



cancer cells. In continuation of our structural modification studies concerning the anthracenone core, the naphtho[2,3-*b*]-thiophene-4(9*H*)-one (**14**) was synthesized as a starting material for the synthesis of 9-benzylidene-naphtho[2,3-*b*]thiophen-4-ones (**15**, Chart 2). The bioisosteric replacement based on the similarity between the physicochemical properties of the benzene and the thiophene is a well fundamented and important strategy of molecular modification leading to compounds with potentially distinct phamacodynamic and pharmacokinetic profiles.[12] Also, thiophenes have emerged as important structural motifs found in several recently described antimitotic agents.[7,13,14]

Herein, we describe the synthesis and biological evaluation of 9-benzylidene-naphtho[2,3-*b*]thiophen-4-ones as a novel class of tubulin polymerization inhibitors. For antiproliferative action, selected compounds were evaluated in the XTT assay. Several compounds strongly inhibited the growth of various tumor cell lines at nanomolar concentrations, acted in a cell-cycle-dependent manner, and were found to be potent inhibitors of tubulin polymerization in the turbidity assay. Antitubulin activities were comparable to those of the reference compounds, such as nocodazole, podophyllotoxin, and colchicine, as determined in the same assay. In vivo antitumor activities were evaluated in syngeneic mice inoculated with P388 tumor fragments.

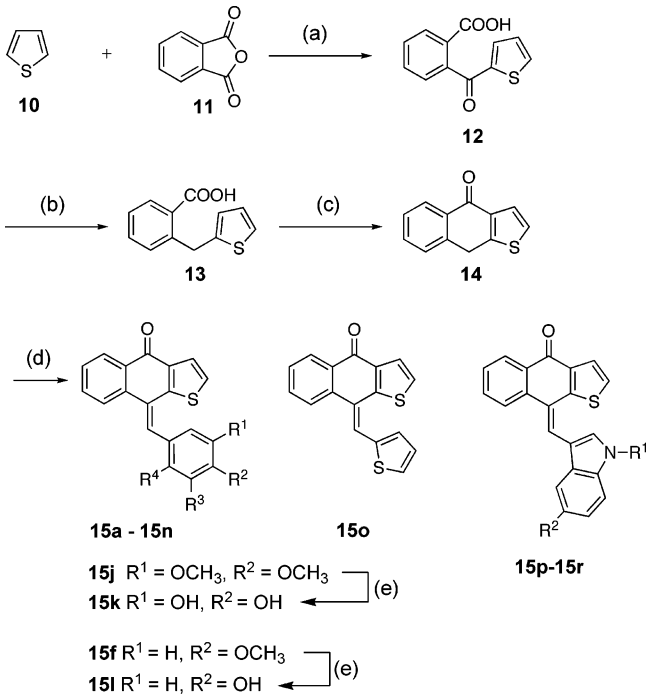
Chemistry

The general method for the synthesis of 9-benzylidene-naphtho[2,3-*b*]thiophen-4-ones is outlined in Scheme 1. Naphtho[2,3-*b*]thiophene-4(9*H*)-one (**14**) was prepared according to a method published by MacDowell and Wisowaty,[15] thus avoiding the formation of constitutional isomers regarding the position of the ketone function in **14**. Friedel−Crafts acylation of thiophene (**10**) with phthalic anhydride (**11**) afforded the carboxylic acid **12**[16−18] in a 70% yield. Reduction of the ketone functional group using zinc dust and aqueous ammonia gave the intermediate **13**,[19] which was reacted in a cyclization via the acid chloride to obtain **14**[20] in 50% yield after purification. A condensation reaction of the structurally related 9(10*H*)-anthracenone with aromatic aldehydes is generally applicable.[21] Using this method, the desired 9-benzylidene-naphtho[2,3-*b*]-thiophen-4-ones **15a**−**15r** were obtained by an aldol-type condensation reaction of **14** with appropriately substituted aromatic aldehydes under basic conditions in the presence of pyridine/piperidine (method A, Experimental Section).[22,23] Alternatively, gaseous hydrogen chloride was used for the condensation reaction (method B).[24] The starting aldehydes were obtained from commercial sources or prepared according to the literature. For the preparation of compounds **15k** and **15l**, ether groups of **15j** and **15f** were cleaved by boron tribromide in dichloromethane.

The constitution of the target compounds suggested the formation of geometric isomers (Chart 2). The ^{1}H NMR analysis, including two-dimensional techniques (^{1}H−^{1}H NOESY, ^{1}H−^{1}H ROESY), clearly assigned (*Z*)-geometric isomers as the main product, and (*E*)-geometric isomers were ranging between 10 and 20%. Although diastereomers can often be easily separated by chromatography or recrystallization, separation of the geometric isomers failed.

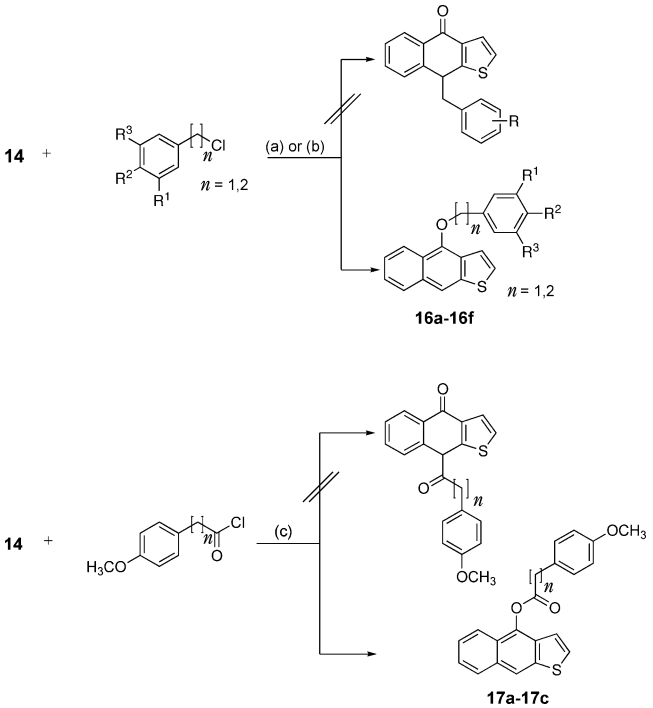
Attempts to alkylate[11,25] **14** at C-9 were unsuccessful (Scheme 2). Interestingly, in contrast to the previously described C-10 alkylation of 9(10*H*)anthracenone,[11] reaction of **14** with the corresponding benzyl chlorides in the presence of potassium

Scheme 1[a]



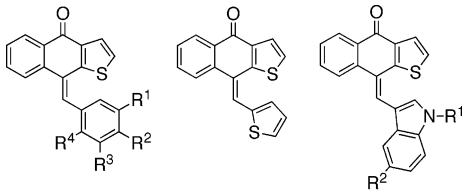
[a] R^1−R^4 are defined in Table 1. Reagents and conditions: (a) $AlCl_3$, CH_2Cl_2, rt; (b) Zn°, conc. NH_3, reflux, 48 h; (c) PCl_5, 0 °C → reflux, $SnCl_4$, 0−4 °C; (d) substituted benzaldehyde, acidic (gaseous HCl) or basic (pyridine/piperidine) conditions; (e) BBr_3, CH_2Cl_2, −78 °C, N_2.

Scheme 2[a]



[a] R^1−R^3 are defined in Table 2. Reagents and conditions: (a) BnCl, K_2CO_3, DMF, reflux; (b) BnCl, K_2CO_3, KI, acetone, reflux; (c) pyridine, toluene, 80 °C, N_2.

Table 1. Antiproliferative Activity of **15a**−**15r** against K562 Cells and Antitubulin Activities



15a-15n 15o 15p-15r

cmpd	R^1	R^2	R^3	R^4	K562 IC_{50}[a] [μM]	ITP[b] IC_{50} [μM]
14					7	
15a	OH	OCH_3	H	H	0.23	1.4
15b	OCH_3	OH	H	H	0.10	0.88
15c	OCH_3	OCH_3	OCH_3	H	0.60	7.4
15d	OCH_3	OH	OCH_3	H	0.05	0.38
15e	H	OCH_3	OH	OCH_3	0.08	0.50
15f	H	OCH_3	H	H	0.44	3.3
15g	H	H	H	H	0.33	6.2
15h	H	NO_2	H	H	0.72	>10
15i	Cl	H	Cl	H	0.42	ND
15j	OCH_3	OCH_3	H	H	0.32	ND
15k	OH	OH	H	H	0.27	ND
15l	H	OH	H	H	0.81	ND
15m	Br	OH	Br	H	6.69	ND
15n	H	OCH_3	H	OH	0.23	0.77
15o					0.39	1.34
15p	H	H			0.72	2.80
15q	CH_3	H			0.25	ND
15r	H	OCH_3			0.24	ND
colchicine					0.02	1.4
nocodazole					ND	0.76
podophyllotoxin					ND	0.35
vinblastine sulfate					0.001	0.13
adriamycin					0.01	ND

[a] IC_{50}, concentration of drug required for 50% inhibition of cell growth (K562). Cells were treated with drugs for 2 days. IC_{50} values are the means of at least three independent determinations (SD < 10%). [b] ITP = inhibition of tubulin polymerization; IC_{50} values were determined after 20 min at 37 °C and represent the concentration for 50% inhibition of the maximum tubulin assembly rate.

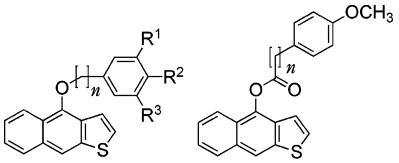
carbonate in DMF afforded *O*-alkylated enols **16a**−**16f** instead of *C*-alkylation. Keto−enol tautomerism in the thiophene analogues of anthrone has been described,[20] and the formation of compounds **16a**−**16f** is suggested to be due to a base-catalyzed enolization of **14**. Attempts to obtain the *C*-alkylated compounds by using benzyl bromides or by alkylating naphtho-[2,3-*b*]thiophen-4(9*H*)-one monoanion in aqueous alkaline solution[26] failed.

Moreover, reaction of **14** with acyl chlorides gave phenolic esters **17a**−**17c** instead of C-9-acylation via the carbanion (Scheme 2).

Biological Results and Discussion

In Vitro Cell Growth Inhibition Assay. The compounds were preliminarily screened for antiproliferative activity against the human chronic myelogenous leukemia cell line K562.[27] Cell proliferation was determined directly by counting the cells with a hemocytometer after 48 h of treatment. The structures of **15a**−**15r** and the growth inhibitory data are listed in Table 1. The observed antiproliferative activities depended on the substitution pattern of the benzylidene part, and almost all compounds showed IC_{50} values in the range of <1 μM, with the exception of **15m**. 9-[(4-Hydroxy-3,5-dimethoxy-benzylidene]-naphtho-[2,3-*b*]thiophen-4(9*H*)-one (**15d)** displayed strong antiproliferative activity (IC_{50} K562: 0.05 μM). It was the most active

Table 2. Antiproliferative Activity of **16a**−**16f** and **17a**−**17c** against K562 Cells and Antitubulin Activities



16a-16f 17a-17c

cmpd	*n*	R^1	R^2	R^3	K562 IC_{50}[a] [μM]	ITP[b] IC_{50} [μM]
16a	1	H	OCH_3	H	0.25	1.52
16b	1	H	H	H	2.38	ND
16c	1	H	NO_2	H	3.13	ND
16d	1	OCH_3	OCH_3	OCH_3	8.95	>10
16e	1	OCH_3	OCH_3	H	0.90	3.98
16f	2	H	OCH_3	H	5.3	>10
17a	0				>90	>10
17b	1				15	>10
17c	2				>90	ND

[a] IC_{50}, concentration of drug required for 50% inhibition of cell growth (K562). Cells were treated with drugs for 2 days. IC_{50} values are the means of at least three independent determinations (SD < 10%). [b] ITP = inhibition of tubulin polymerization; IC_{50} values were determined after 20 min at 37 °C and represent the concentration for 50% inhibition of the maximum tubulin assembly rate.

compound in this assay, being slightly less potent than adriamycin (IC_{50} K562: 0.01 μM) and colchicine (IC_{50} K562 0.02 μM). Similar activity was found for the constitutional isomer **15e** (IC_{50} K562 0.08 μM). In addition, compound **15b** was also found to be active with an IC_{50} value of 0.1 μM. Noteworthy, compound **15a**, bearing an isovanilline partial structure, was determined to be clearly less potent than the recently described close analogue **9** (**15a**, 0.23 μM vs 0.02 μM for **9**). Replacement of methoxy groups by bromo atoms turned out to be detrimental to potency in the case of **15m** (IC_{50} K562: 6.7 μM). Introduction of chloro (**15i**) or nitro substituents (**15h**) also resulted in compounds with reduced antiproliferative activity compared with those bearing methoxy and/or hydroxy substituents. The unsubstituted compound **15g** showed modest antiproliferative activity (IC_{50} K562: 0.33 μM). Also, the dimethoxy-substituted derivative **15j** had an IC_{50} value of 0.32 μM, thus being 5-fold less active than **15d**. Interestingly, introduction of a 3,4,5-trimethoxyphenyl group in **15c**, a well-defined pharmacophore found in cytotoxic agents such as colchicine, combretastatin A-4, and podophyllotoxin, led to a 10-fold decrease in growth inhibitory properties as compared with **15d**. Compounds **15o**−**15r** showed activities in the submicromolar range, with IC_{50} values ranging from 0.2 to 0.7 μM. The indolyl-based compounds **15q** and **15r** were potent antiproliferative agents, displaying IC_{50} values in the range of 0.25 μM, but were substantially less active than **15d**. In addition, naphtho[2,3-*b*]-thiophen-4(9*H*)-one (**14**) displayed only weak activity, showing that the benzylidene structure is closely related to the antiproliferative activity.

Combretastatin A-4-like benzyl ether compounds have been described as potent antiproliferative agents against the K562 cell line.[28] By contrast, our benzyl ether compounds, with the exception of **16a** (IC_{50} K562: 0.25 μM), revealed weak inhibitory effect on cell growth (Table 2, **15g** vs **16b**, **15h** vs **16c**, **15c** vs **16d)**. Again, if the terminal aromatic ring was substituted with trimethoxy (**16d**), growth inhibition decreased as compared to that of **16a**. Furthermore, chain elongation as exemplified by **16f** did not lead to an improved efficacy in growth inhibition. Ester compounds **17a**−**17c** proved to be completely inactive.

Table 3. Antiproliferative Activity of **15d**, **15e**, and **16a** against Various Cell Lines after 48 h Treatment

	IC_{50}[a] [μM]					
					RKOp27^{kip1} (human colon adenocarcinoma)	
cmpd	KB/HeLa (cervix)	SKOV3 (ovary)	SF268 (glioma)	NCI-H460 (lung)	not induced	induced
15d	0.05	0.07	0.07	0.05	0.03	>9
15e	0.19	0.04	0.05	0.28	0.04	>9
16a	0.47	0.27	0.43	0.98	0.28	>9
paclitaxel	0.01	0.01	0.01	0.01	0.01	>3
nocodazole	0.14	0.17	0.30	0.15	0.11	>10
colchicine	0.03	0.05	0.05	0.07	0.02	>10

[a] IC_{50} values were determined from XTT proliferation assays after incubation with test compound for 48 h. All experiments were performed at least in two replicates ($n = 2$), and IC_{50} data were calculated from dose−response curves by nonlinear regression analysis.

Table 4. Antiproliferative Activity of **15d** and **15e**, Paclitaxel, Nocodazole, and Vindesine against Tumor Cell Lines with Different Resistance Phenotypes (XTT Assay)

	IC_{50}[a] [μM]					
cmpd	LT12	LT12 MDR	L1210	L1210 VCR	P388	P388 ADR
15d	0.13	0.17	0.34	0.68	0.05	0.22
15e	0.18	0.19	0.13	0.17	0.14	0.16
Paclitaxel	0.006	0.40	0.06	>5	0.04	>5
nocodazole	0.04	0.07	0.05	0.09	0.08	0.17
vindesine	0.001	0.26	0.02	>5	0.01	1.10

[a] IC_{50} values were determined from XTT proliferation assays after incubation with test compound for 48 h. All experiments were performed at least in two replicates ($n = 2$), and IC_{50} data were calculated from dose−response curves by nonlinear regression analysis.

Effect on Growth of Different Tumor Cell Lines. To further evaluate the antiproliferative properties, the effect of the most active compounds **15d** and **15e** against a panel of five tumor cell lines derived from solid tumors was measured by cellular metabolic activity using the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) assay.[29] Both compounds were slightly more potent than nocodazole and showed excellent activities with IC_{50} values in the range <0.1 μM toward several proliferating cell lines (Table 3). Moreover, the compounds were not active against RKO cells (human colon adenocarcinoma) with ectopic inducible expression of cyclin-dependent kinase inhibitor p27^{kip1}.[30] By contrast, growth of proliferating RKO cells was strongly inhibited by **15d** and **15e** with IC_{50} values of 0.03 and 0.04 μM, respectively, indicating activity toward cycling cells. Considering 4-(4-methoxy-benzyloxy)-naphtho[2,3-*b*]thiophene **16a**, as expected, this compound proved to be clearly less active than **15d** and **15e** (Table 3). A major obstacle to the treatment of many human cancers is the development of multiple drug resistance (MDR) in patients and a loss of efficacy over time, meaning that cancer cells do not respond to chemotherapy by developing a broad spectrum resistance to several anticancer drugs, among them antitubulin agents.[31,32] Multiple drug resistance is mediated, among other factors, by overexpression of transmembrane cellular pumps, such as the 170 kDa P-glycoprotein (pgp),[33] encoded by the *mdr*1 gene and the 180 kDa MDR protein (MRP).[34] Important aspects concerning the key mechanisms of antimicrotubule drug resistance have recently been reviewed.[35] The antiproliferative activity of **15d** and **15e** against tumor cell lines with different resistance phenotypes was evaluated in an XTT-based assay. On the whole, as documented by the IC_{50} data (Table 4), **15d** and **15e** were effective against the cell lines tested and retained activity in cell lines with various multiple drug resistance phenotypes. This feature was distinct from paclitaxel and vindesine because LT12MDR, L1210VCR, and P388ADR cell lines were more resistant to these chemotherapeutics than the nonresistant cell lines. Therefore, the naphtho[2,3-*b*]thiophen-4(9*H*)-ones **15d** and **15e** are inhibitors of tumor cell proliferation and are not subject to resistance by overexpression of pgp170.

Effect on Cell-Cycle Progression. The strong antiproliferative activity of **15d** on cancer cells prompted us to test its effects on the cell-cycle progression. We incubated K562 cells with **15d** in a concentration-dependent manner for 24 h and obtained the cell-cycle profile as shown in Figure 1. The cell-cycle-dependent DNA content was determined by flow cytometry using propidium iodide in permeabilized cells. It became evident that compound **15d** accumulated 50% cells at G2/M in the range of 0.05 μM, suggesting that this compound arrested the cell cycle at the G2/M-phase. For a thorough comparison of **15d** with known G2/M cell-cycle inhibitors, we used an established KB/HeLa (human cervical epitheloid carcinoma) cell-based assay system. Subconfluent KB/HeLa cells were exposed to test compounds, and the percentage of cells in G2/M phase after 24 h was plotted against different concentrations of the compounds. The concentration for 50% cells arrested in G2/M phase was found to be 0.1 μM for **15d** (Table 5), thus being virtually as active as nocodazole (EC_{50} 0.09 μM) but being more active than the recently described **9** (EC_{50} 0.2 μM). In summary, the effect of compound **15d** on cell-cycle progression correlated well with its strong antiproliferative and antitubulin activities and is similar to that observed for the majority of antimitotic agents.

In Vitro Tubulin Polymerization Assays. We selected 17 compounds to determine their antitubulin polymerization activities as presented in Table 1 and Table 2. The turbidity assay, a spectrophotometric assay based on light reflection by microtubules, provides a covenient in vitro method to pre-investigate the effect of a drug on tubulin polymerization/depolymerization. According to the temperature-dependent equilibrium between α/β-tubulin and microtubules, physiological temperature (37 °C) favors polymerization of tubulin dimers into microtubules, which is detected by an increase in turbidity. Depolymerization is induced by shifting the temperature to 2 °C. If a drug binds to or interferes with tubulin, the assembly steady-state level is decreased for tubulin polymerization inhibitors, as exemplified for **15d** (Figure 2). The results obtained with the test agents are summarized in Table 1. For comparison, the data of the potent antimitotic compounds colchicine, podophyllotoxin, nocodazole, and vinblastine are also presented. In general, inhibition of tubulin polymerization correlated well with the drug growth inhibition. For instance, compounds **15b**, **15d**, **15e**, and **15n** were found to be strong inhibitors of tubulin polymerization ($IC_{50} \leq 1.0$ μM), as did the reference drugs, with the exception of colchicine (1.4 μM). Also, these compounds exhibited stronger antiproliferative activities than the other benzylidene-naphtho[2,3-*b*]thiophen-4-ones tested. The least growth inhibitory compounds had only weak inhibitory effects on tubulin

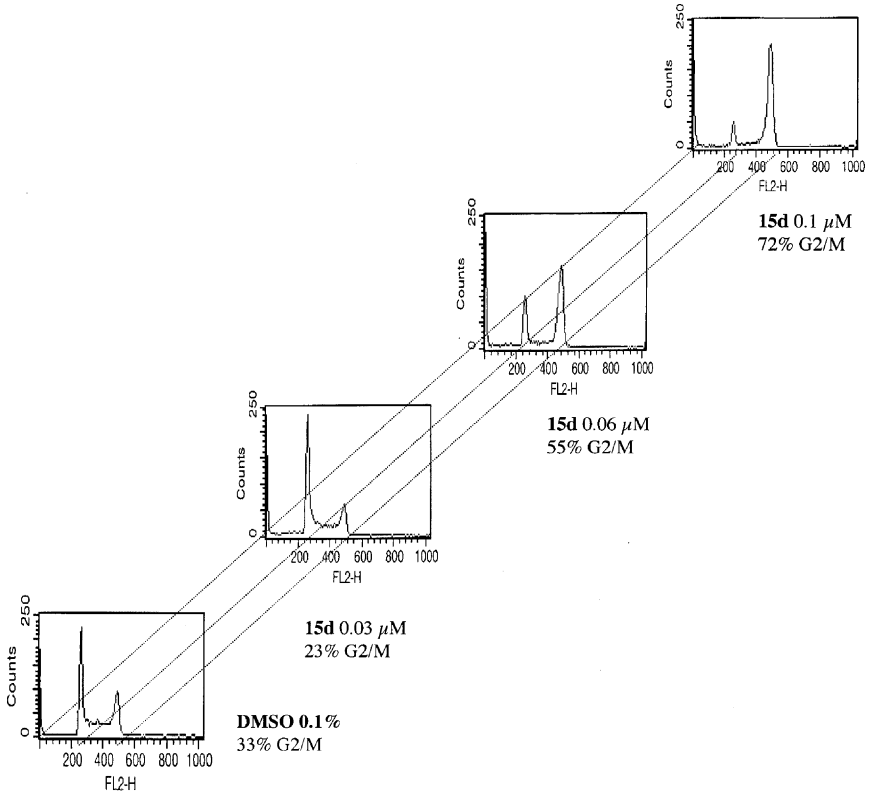


Figure 1. Induction of cell-cycle arrest by **15d**. K562 cells were treated with **15d** in a concentration-dependent manner. Then, the cells were collected and cell-cycle distribution was measured by flow cytometry.

Table 5. Cell-Cycle Analysis of KB/HeLa Cells Treated with **15d** and Reference Compounds Vincristine, Colchicine, Paclitaxel, and Nocodazole

	15d	colchicine	nocodazole	paclitaxel	vincristine
EC_{50}[a] [nM]	98	14	91	49	2.4

[a] EC_{50} values were determined from dose−response cell-cycle analysis experiments and represent the concentration for 50% cells arrested in G2/M phase after 24 h. All experiments were performed at least in two replicates ($n = 2$), and IC_{50} data were calculated from dose−response curves by nonlinear regression analysis (GraphPad Prism).

polymerization (IC_{50} values ranging from 2.8 to >10 μM). The syringaldehyde-derived compound **15d** (IC_{50} 0.38 μM) was as antitubulin-active as podophyllotoxin. It was again found to be the most active compound in this assay. Also, its isomer **15e** proved to be strongly active (IC_{50} 0.05 μM) and exhibited virtually the same activity as **15d**. This indicates that a hydroxy group being flanked by methoxy groups plays an essential role to exhibit strong antitubulin and antiproliferative activities within the benzylidene-naphtho[2,3-*b*]thiophen-4-one series of compounds. Again, the 4-hydrox-3,5-dimethoxyphenyl fragment is a much more active moiety than 4-methoxy (**15f**) or 3-hydroxy-4-methoxy (**15a**) in this series of tubulin polymerization inhibitors. This is in contrast to the previously described series of 10-benzylidene-9(10*H*)-anthracenones,[11] with the isovanillic **9** (Chart 2) being the most active representative. Compound **15n** was as good a tubulin polymerization inhibitor as nocodazole, whereas only weak inhibitory activity was observed for **15c** (IC_{50} 7.4 μM) and **15g** (IC_{50} 6.2 μM). It is interesting to note that the trimethoxyphenyl ring in **15c**, a well-defined pharmacophore for the inhibition of tubulin polymerization found in colchicine, combretastatin A-4, and podophyllotoxin, led to a 15- to 20-fold decrease in the antitubulin activity assay as compared with that of **15d**. This SAR on the terminal phenyl ring was consistent with the results from the growth inhibitory assay. Thiophene and especially indole have emerged as

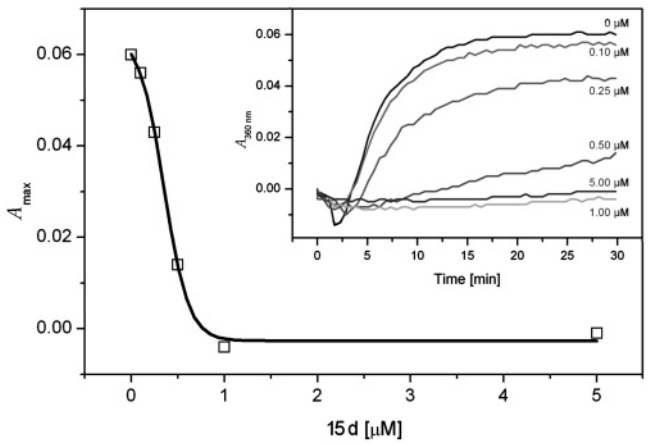


Figure 2. Inhibition of in vitro microtubule assembly by various concentrations of **15d**; turbidity was measured at 360 nm (MTP 1 mg/mL). Tubulin assembly in the absence of inhibitor gave the control readings. IC_{50} values were determined after 20 min at 37 °C and represent the concentration for 50% inhibition of the maximum tubulin assembly rate.

important structural motifs found in several recently described tubulin polymerization inhibitors.[36−38] We found that the indolyle-based compound **15p** showed moderate activity concerning the tubulin polymerization inhibition, whereas **15o** was as active as colchicine. The benzyl ether group has been used as an acyclic linker to replace the *cis*-double bond of CA-4.[28] Within the 4-benzyloxy-naphtho[2,3-*b*]thiophene series of compounds, **16a** showed almost the same activity as colchicine (IC_{50} 1.4 μM), whereas **16d** was inactive (Table 2). Also, ester compounds **17a** and **17b** did not show any appreciable activity as inhibitors of tubulin polymerization.

Microtubule-destabilizing drugs often have specific microtubule binding sites. Therefore, we assessed the capability of **15d** and **15e** to compete with either colchicine or paclitaxel for

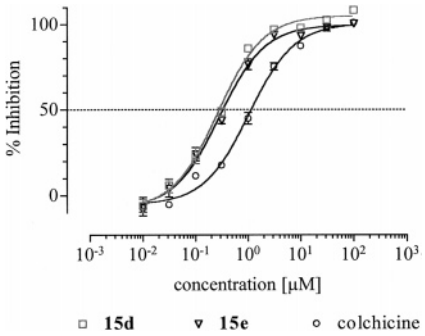


Figure 3. [^{3}H]-Colchicine competition binding assay of **15d**, **15e**, and colchicine. Radiolabeled colchicine, unlabeled compound, and biotin-labeled tubulin were incubated together for 2 h at 37 °C.

binding to tubulin using a competitive scintillation proximity assay.[39] We found that **15d** and **15e** competitively inhibited [^{3}H]colchicine binding to biotinylated tubulin (Figure 3) with exceptionally low IC_{50} values of 0.27 (**15d**) and 0.29 μM (**15e**) versus colchicine (1.06 μM). The results were consistent with their growth inhibitory and tubulin polymerization inhibitory activities. However, the compounds did not compete with [^{3}H]paclitaxel. No stabilization of the colchicine binding was observed, as it is documented for vinca site binders.[40,41] Thus, we conclude that binding to tubulin at the colchicine-binding site is highly probable and that the capacity to affect the mitotic spindle contributes to the antiproliferative activity of the compounds.

In Vivo Antitumor Results. Based on the favorable in vitro properties we chose to examine the activity of compound **15d** in a P388 cancer model. Female CD2F1 mice were subcutaneously inoculated with murine P388 tumor fragments of approximately 1 mm^3 at day 0. Treatment was initiated at day 5 after tumor inoculation. Mice were treated intraperitoneally with three doses of **15d** at 100, 50, and 25 mg/kg body weight weekly for three consecutive weeks, for example, on days 5, 12, and 19 after inoculation of the tumor fragments. Subsequently, tumors developed as a lymphoma with metastasis formation in lungs and liver. The prolongation of survival and inhibition of tumor growth were parameters for activity. A prolongation of survival by 30% or more, for example, a T/C-value of 130% or higher was considered to be significantly better than the control. Unfortunately, despite its high in vitro potency, compound **15d** was devoid of any appreciable activity in this model. None of the treatments induced a significant prolongation of survival. The T/C-values obtained were 106, 111, and 83% for 100, 50, and 25 mg/kg, respectively. Furthermore, no effects of treatment on body weights were observed at any of the doses tested (data not shown). However, subcutaneous tumor growth as a further parameter for activity, was significantly inhibited by **15d** at several time points, for example, between days 9 and 14, upon treatment with **15d** at 50 mg/kg. Moreover, compound **15d** at 25 mg/kg body weight significantly inhibited tumor growth at day 7 post inoculation. In our opinion, the disappointing results obtained with **15d** from the P388 cancer model were possibly due to a lack of solubility under physiological conditions. In consequence, we started investigating several approaches on the best compounds **15d** and **15e** to improve the in vivo results by using the reactivity of the phenol function.

Conclusion

A series of novel 9-benzylidene-naphtho-[2,3-*b*]-thiophen-4-ones and related compounds were synthesized. We have identified two representatives, **15d** and **15e**, to be potent antiproliferative agents, inhibitors of tubulin polymerization, and inhibitors of colchicine binding to tubulin. The compounds described in this report are structurally simpler than the well-known vinblastine or the taxoids, chemically stable, and easily accessible. As many other inhibitors of tubulin polymerization, the most active compounds **15d** and **15e** were efficacious in inhibiting tumor cell proliferation with IC_{50} values at the nanomolar level. Structure−activity relationship studies revealed that the syringaldehyde substitution pattern in the terminal phenyl ring plays an integral role for strong inhibition of tumor cell proliferation and inhibition of tubulin polymerization. The most active of the structurally novel 9-benzylidene-naphtho-[2,3-*b*]thiophen-4-ones are potent inhibitors of the colchicine binding to tubulin. The best compounds **15d** and **15e** most likely interact with tubulin at the colchicine site. As with other microtubule-interacting agents, induction of G2/M arrest was demonstrated for **15d**. No growth inhibitory effect was found in cell-cycle-arrested cells. The effectiveness of many clinically useful drugs is limited by the fact that they are substrates for the efflux pumps Pgp170 and MRP. Compounds **15d** and **15e** were found to be equally potent toward parental tumor cell lines and multidrug resistant cell lines. This feature is distinct from those of paclitaxel and vindesine because three cell lines were more resistant to these agents than the nonresistant cell lines. Considering the benzyloxy-naphtho[2,3-*b*]thiophenes, these compounds were less active than the corresponding benzylidenes in terms of cell growth inibition and inhibition of tubulin polymerization. Despite its potent antitubulin activity and cytotoxicities properties, compound **15d** suffered from poor properties in vivo. Nevertheless, due to their attractive in vitro antitumor activities, we believe that the compounds of this structural class are attractive for further structural modifications and may provide a useful template for the design of new antitumoral agents.

Structure−activity relationship studies concerning the pharmacophore requirements for activity are in progress and will be reported in due course.

Experimental Section

Melting points were determined with a Kofler melting point apparatus and are uncorrected. Spectra were obtained as follows: ^{1}H NMR spectra were recorded with a Varian Mercury 400 plus (400 MHz) spectrometer, using tetramethylsilane as an internal standard. Fourier-transform IR spectra were recorded on a Bio-Rad laboratories Typ FTS 135 spectrometer, and analysis was performed with WIN−IR Foundation software. Elemental analyses were performed at the Münster microanalysis laboratory, using a vario EL III CHNOS elemental analyzer (Elementar Analyzensysteme GmbH), and all values were within ±0.4% of the calculated composition. Mass spectra were recorded in the EI mode using a MAT GCQ Finnigan instrument. All organic solvents were appropriately dried or purified prior to use. Aryl aldehydes were obtained from commercial sources. Analytical TLC was done on Merck silica 60 F_{254} alumina coated plates (E. Merck, Darmstadt). Silica gel column chromatography was performed using Acros 60−200 mesh silica gel. All new compounds displayed ^{1}H NMR, FTIR, and MS spectra consistent with the assigned structure. Yields have not been optimized. Chromatography solvent (vol %): EE = ethyl acetate; H = hexane; M = methanol; MC = methylene chloride. Elemental analyses were within ±0.4% of calculated values, except where stated otherwise.

Synthesis of 2-(Thiophene-2-carbonyl)-benzoic Acid (12). A solution of phthalic anhydride (**11**, 29.6 g, 0.2 mol) in 150 mL CH_2Cl_2 was added to a stirred suspension of $AlCl_3$ (58.7 g, 0.44 mol) in dry CH_2Cl_2 (150 mL). The mixture was allowed to stir at rt for 30 min. Thereafter, thiophene (**10**, 0.2 mol, 15.76 mL) was slowly added within 1 h. The mixture was stirred at room

temperature during 3 h, after which the reaction was quenched by addition of 0.2 M HCl (1 L) with stirring for 1 h.

The aqueous layer was extracted with CH_2Cl_2, and the combined organic portions were washed with NaOH (1 N, 3 × 200 mL). The 2-(thiophene-2-carbonyl)-benzoic acid was then precipitated by acidification with HCl 37% (100 mL). The precipitate was filtered, washed with water, and dried. Recrystallization from methanol/water (1:1) afforded colorless crystals (32.48 g, 70%), mp 145−146 °C (lit.[17] 145 °C).

Synthesis of 2-Thiophene-2-yl-methyl-benzoic Acid (13). A stirred suspension of 2-(thiophene-2-carbonyl)-benzoic acid **12** (12 g, 52 mmol), concentrated NH_3 (350 mL), $CuSO_4 \cdot 5H_2O$ (1.8 g), and Zn powder (42 g) was heated to reflux for 48 h. Three portions of concentrated NH_3 (20 mL) were added over this period. The hot solution was filtered and then acidified with HCl 37% while stirring. The white precipitate was filtered and thereafter dried (Dean Stark) to afford colorless crystals (6.77 g, 60%), mp 102−105 °C (lit.[42] 102−103 °C).

Synthesis of Naphtho[2,3-*b*]thiophen-4(9*H*)-one (14). 2-Thiophene-2-yl-methyl-benzoic acid (**13**; 10.0 g, 46 mmol) was suspended in dry benzene (150 mL) under N_2 and cooled to 0 °C. The mixture was stirred, and PCl_5 (9.57 g, 46 mmol) was slowly added over 20 min. The suspension was thereafter heated to reflux until the HCl production stopped (1 h). Then the mixture was cooled to 0−4 °C. After 20 min, a solution of $SnCl_4$ (8.0 mL, 68 mmol) in dry benzene (50 mL) was added dropwise over 30 min. A yellow-green precipitate was formed. The mixture was stirred overnight, poured into a mixture of water (500 mL) and concentrated HCl (50 mL), and extracted with CH_2Cl_2 (3 × 100 mL). The organic phase was washed with water, dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified by chromatography using silica gel to afford pale yellow crystals (4.59 g, 50%), mp 128 °C (lit.[15] 130−132 °C).

General Procedure for the Preparation of 9-Benzylidene-naphtho[2,3-*b*]thiophen-4-ones 15a−15j and 15m−15r. Method A. Naphtho[2,3-*b*]thiophen-4(9*H*)-one (**14**; 1.0 g, 5 mmol) and the appropriately substituted aromatic aldehyde (5 mmol) were suspended in 30 mL of dry pyridine under N_2. Piperidine (5 mL) was added, and the mixture was thereafter heated on an oil bath (130 °C) until the reaction was completed (TLC control). Then, the reaction mixture was cooled to room temperature and poured on a mixture of water (300 mL) and 50 mL of 6 N HCl. The precipitate was extracted with CH_2Cl_2 (3 × 100 mL), and the organic phase was dried over Na_2SO_4 and then concentrated in vacuo. Finally, the residue was purified by chromatography using silica gel.

Method B. Naphtho[2,3-*b*]thiophen-4(9*H*)-one (**14**; 1.0 g, 5 mmol) and the substituted benzaldehyde (5 mmol) were dissolved in absolute ethanol (50 mL) at room temperature. Then, the suspension was saturated with gaseous HCl for 5 min. The mixture became dark and was thereafter heated to reflux for 1 h (TLC control). Then, the reaction mixture was cooled to room temperature, poured into water (300 mL), and extracted with CH_2Cl_2 or ethyl acetate (phenolic compounds). Solvents were evaporated at reduced pressure, and the residue was purified by column chromatogaphy using silica gel. All products were obtained as solids.

Cleavage of Ethers with Boron Tribromide (15k, 15l). The substrate (0.58 mmol) was dissolved in CH_2Cl_2 (10 mL) and cooled to −78 °C. A solution of 1 M BBr_3 in CH_2Cl_2 (5 mL, 5 mmol) was added dropwise with stirring. The stirring was continued overnight while the mixture was allowed to warm to rt. Then, the reaction was terminated by addition of water (300 mL), and the product was subsequently extracted with ethyl acetate. The extract was dried with Na_2SO_4, and the solvent was then removed in vacuo. The residue was then chromatographed on a column of SiO_2 using ethyl acetate as eluent.

9-[(3-Hydroxy-4-methoxy)-benzylidene]-naphtho[2,3-*b*]-thiophen-4(9*H*)-one (15a). Purification by silica gel chromatography (MC) afforded **15a** as orange crystals (method A, 14% yield; method B, 29% yield): mp 179−182 °C.

9-[(4-Hydroxy-3-methoxy)-benzylidene]-naphtho[2,3-*b*]-thiophen-4(9*H*)-one (15b). Purification by silica gel chromatography (MC) afforded **15b** as an orange solid (method A, 16% yield): mp 175−178 °C.

9-[(3,4,5-Trimethoxy)-benzylidene]-naphtho[2,3-*b*]thiophen-4(9*H*)-one (15c). Purification by silica gel chromatography (MC/H 8:2) afforded **15c** as light-yellow crystals (method A, 17% yield): mp 196−199 °C.

9-[(4-Hydroxy-3,5-dimethoxy-benzylidene]-naphtho[2,3-*b*]-thiophen-4(9*H*)-one (15d). Purification by silica gel chromatography (MC) afforded **15d** as an orange solid (method A, 29% yield; method B, 67% yield): mp 192−195 °C.

9-[(3-Hydroxy-2,4-dimethoxy)-benzylidene]-naphtho[2,3-*b*]-thiophen-4(9*H*)-one (15e). Purification by silica gel chromatography (MC) afforded **15e** as a light-yellow solid (method A, 16% yield; method B, 53% yield): mp 210−212 °C.

9-[(4-Methoxy)-benzylidene]-naphtho[2,3-*b*]thiophen-4(9*H*)-one (15f). Purification by silica gel chromatography (MC) afforded **15f** as yellow crystals (method A, 7% yield; method B, 4% yield): mp 114−116 °C.

9-Benzylidene-naphtho[2,3-*b*]thiophen-4(9*H*)-one (15g). Purification by silica gel chromatography (MC/H 5:2) afforded **15g** as light-yellow needles (method A, 15% yield): mp 135−137 °C.

9-[(4-Nitro)-benzylidene]-naphtho[2,3-*b*]thiophen-4(9*H*)-one (15h). Purification by silica gel chromatography (MC/H 1:1) afforded **15h** as yellow crystals (method A, 8% yield): mp 200−204 °C.

9-[(3,5-Dichloro)-benzylidene]-naphtho[2,3-*b*]thiophen-4(9*H*)-one (15i). Purification by silica gel chromatography (MC) afforded **15i** as a yellow powder (method B, 50% yield): mp 219−221 °C.

9-[(3,4-Dimethoxy)-benzylidene]-naphtho[2,3-*b*]thiophen-4(9*H*)-one (15j). Purification by silica gel chromatography (MC/H 2:1) afforded **15j** as a yellow powder (method B, 37% yield): mp 190−192 °C.

9-[(3,4-Dihydroxy)-benzylidene]-naphtho[2,3-*b*]thiophen-4(9*H*)-one (15k). Purification by silica gel chromatography (MC/EE 1:1) afforded **15k** as a yellow powder (33% yield): mp 229 °C.

9-[(4-Hydroxy)-benzylidene]-naphtho[2,3-*b*]thiophen-4(9*H*)-one (15l). Purification by silica gel chromatography (MC/H 8:2) afforded **15l** as orange crystals (38% yield): mp 240 °C.

9-[(3,5-Dibromo-4-hydroxy)-benzylidene]-naphtho[2,3-*b*]-thiophen-4(9*H*)-one (15m). Purification by silica gel chromatography (MC) afforded **15m** as an orange powder (method B, 41% yield): mp 240−243 °C.

9-[(2-Hydroxy-4-methoxy)-benzylidene]-naphtho[2,3-*b*]-thiophen-4(9*H*)-one (15n). Purification by silica gel chromatography (MC/M 9:1) afforded **15n** as an orange powder (method B, 48% yield): mp 260−262 °C.

9-Thiophen-2-yl-methylene-9*H*-naphtho[2,3-*b*]thiophen-4-one (15o). Purification by silica gel chromatography (MC/H 1:1) afforded **15o** as a light-yellow powder (method A, 2% yield; method B, 10% yield): mp 142−144 °C.

9-(1*H*-Indol-3-yl-methylene)-9*H*-naphtho[2,3-*b*]thiophen-4-one (15p). Purification by silica gel chromatography (MC) afforded **15p** as a wine-red powder (method A, 8% yield): mp 240−242 °C.

9-(1-Methyl-1*H*-indol-3-yl-methylene)-9*H*-naphtho[2,3-*b*]-thiophen-4-one (15q). Purification by silica gel chromatography (EE/H 3:7) afforded **15q** as a wine-red powder (method A, 2% yield; method B, 25% yield): mp 244−245 °C.

9-(5-Methoxy-1*H*-indol-3-yl-methylene)-9*H*-naphtho[2,3-*b*]-thiophen-4-one (15r). Purification by silica gel chromatography (MC) afforded **15r** as a wine-red powder (method A, 7% yield): mp 116−118 °C.

General Procedure for the Preparation of 4-Benzyloxy-naphtho[2,3-*b*]thiophenes 16a-16f. Method A. To a suspension of **14** (1 g, 5 mmol) and dry K_2CO_3 (3.04 g, 22 mmol) in absolute DMF (30 mL) was added dropwise a solution of the benzyl halide (11 mmol), and the mixture was refluxed under N_2 until the reaction was completed (TLC control). The reaction mixture was then filtered, and the filtrate was poured onto water (250 mL) and extracted with CH_2Cl_2 (3 × 75 mL). The combined organic phases

were dried over Na_2SO_4 and evaporated, and the residue was purified by chromatography.

Method B.[43,44] Naphtho[2,3-*b*]thiophen-4(9*H*)-one **14 (**1 g, 5 mmol), dry K_2CO_3 (1.38 g, 10 mmol), and KI (0.1 g) were suspended in absolute acetone (60 mL) under N_2. The benzyl halide was added (5 mmol), and the mixture was refluxed (TLC control). After the reaction was completed, the mixture was filtered, treated with water (250 mL), extracted with CH_2Cl_2, dried over Na_2SO_4, and then evaporated. The residue was purified by chromatography.

Preparation of Benzyl Halides. Benzyl halides were commercially available or prepared from the corresponding alcohols by following standard literature procedures.[45−48]

4-(4-Methoxy-benzyloxy)-naphtho[2,3-*b*]thiophene (16a). Purification by silica gel chromatography (MC/H 1:1) afforded **16a** as white crystals (method A, 27% yield; method B, 12% yield): mp 119−121 °C.

4-(Benzyloxy)-naphtho[2,3-*b*]thiophene (16b). Purification by silica gel chromatography (MC/H 1:3) afforded **16b** as a white powder (method A, 30% yield): mp 38−44 °C.

4-(4-Nitro-benzyloxy)-naphtho[2,3-*b*]thiophene (16c). Purification by silica gel chromatography (MC/H 1:2) afforded **16c** as yellow crystals (method B, 4% yield): mp 148−151 °C.

4-(3,4,5-Trimethoxy-benzyloxy)-naphtho[2,3-*b*]thiophene (16d). Purification by silica gel chromatography (MC/H 1:1) afforded **16d** as white needles (method A, 23% yield): mp 60−62 °C.

4-(3,4-Dimethoxy-benzyloxy)-naphtho[2,3-*b*]thiophene (16e). Purification by silica gel chromatography (MC/H 8:2) afforded **16e** as white crystals (method A, 25% yield): mp 86−87 °C.

4-[2-(4-Methoxy-phenyl)-ethoxy]-naphtho[2,3-*b*]thiophene (16f). Purification by silica gel chromatography (MC/H 1:1) afforded **16f** as white crystals (method A, 17% yield): mp 123−124.

General Procedure for the Preparation of 4-Acyl Derivatives of Naphtho[2,3-*b*]-thiophen-ol 17a−c.[25,49] To a solution of **14** (1.0 g, 5 mmol) in absolute toluene (50 mL) and dry pyridine (0.40 mL, 5 mmol) was added dropwise a solution of the acid chloride (5 mmol) dissolved in toluene (5 mL) under N_2. The mixture was stirred at 80 °C until the reaction was completed (TLC control), cooled, and filtered, and the filtrate was evaporated. The residue was chromatographed on silica gel to afford the title compounds.

Preparation of Acyl Chlorides. Acid chlorides were commercially available or prepared from the corresponding acids by following standard literature procedures.[50,51] In most cases, the crude products were used for acylation.

4-Methoxy-benzoic Acid-naphtho[2,3-*b*]thiophen-4-yl-ester (17a). The title compound was obtained from naphtho[2,3-*b*]-thiophen-4(9*H*)-one (0.5 g, 2.5 mmol) and 4-methoxybenzoic acid chloride (0.64 g, 3.75 mmol), following the general procedure described above. Purification by silica gel chromatography (MC) afforded **17a** as white crystals (53% yield): mp 230−235 °C.

(4-Methoxy-phenyl)-acetic Acid-naphtho[2,3-*b*]thiophen-4-yl-ester (17b). The title compound was obtained as a white solid from naphtho[2,3-*b*]thiophen-4(9*H*)-one (**14**, 0.5 g, 2.5 mmol) and 4-methoxyphenylacetyl chloride (0.69 g, 0.57 mL, 3.75 mmol), following the general procedure described above. Purification by silica gel chromatography (MC/H 8:2) afforded **17b** as colorless crystals (74% yield): mp 125−127 °C.

3-(4-Methoxy-phenyl)-propionic Acid-naphtho[2,3-*b*]thiophen-4-yl-ester (17c). Purification by silica gel chromatography (MC/H 1:2) afforded **17c** as a yellow solid (38% yield). The title compound was obtained as white crystals from naphtho[2,3-*b*]thiophen-4(9*H*)-one (**14**, 1 g, 5 mmol) and 3-(4-methoxyphenyl)propionic acid chloride[52] (1.48 mL, 10 mmol), following the general procedure described above (38% yield): mp 140−142 °C.

Biological Assay Methods. Materials. Vinblastine sulfate was obtained from Calbiochem (art. no. 677175). Chemicals were dissolved in methanol/DMSO 1:1. Vinblastine sulfate was dissolved in methanol/water 1:1.

Cells and Culture Conditions. Human chronic myelogenous K562 leukemia cells were obtained from DSMZ, Braunschweig, Germany, and cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS, Biochrom KG), streptomycin (0.1 mg/mL), penicillin G (100 units/mL), and L-glutamine (30 mg/L) at 37 °C in 5% CO_2. Cell line SF268 (central nerval system carcinoma) was obtained from NCI. The human tumor cell lines KB/HeLa (cervical carcinoma), SKOV3 (ovarian adenocarcinoma), NCI-H460, and L1210 (murine leukemia) were obtained from ATCC. The RKO human colon adenocarcinoma cells containing an ecdysone-inducible expression vector of $p27^{kip1}$ were described recently.[53]

The $L1210^{VCR}$ cell line was described recently.[54] Rat LT12 cells, the LT12/MDR subline as well as P388 cells and the P388/Adr subline were provided by Dr. Nooter (Univ. Hosp., Rotterdam, NL).

Assay of Cell Growth. K562 cells were plated at 2×10^5 cells/mL in 48-well dishes (Costar, Cambridge, MA). Untreated control wells were assigned a value of 100%. Drugs were made soluble in DMSO/methanol 1:1, and control wells received equal volumes (0.5%) of vehicle alone. To each well was added 5 μL of drug, and the final volume in the well was 500 μL. Cell numbers were counted with a Neubauer counting chamber (improved, double grid) after treatment with chemicals for 48 h. Each assay condition was prepared in triplicate, and the experiments were carried out three times. IC_{50} values are the concentration at which cell growth was inhibited by 50%. To calculate the inhibition of growth, the number of cells at time 0 was first subtracted. The adjusted cell number was calculated as a percentage of the control, which was the number of cells in wells without the addition of compound.

XTT Assay. The XTT assay was used to determine proliferation by quantification of cellular metabolic activity.[29] IC_{50} values were obtained by nonlinear regression (GraphPad Prism). The various tumor cell lines were seeded in microtiter plates in a density of 1×10^3 to 8×10^4 cells/well in 125 μL for logarithmic cell growth and were incubated with different concentrations of cytotoxic agents for 48 h.

In RKOp27^{Kip1} cells, expression of $p27^{Kip1}$ was induced by 3 μM ponasterone A in 24 h, leading to an arrest of these cells in the G1 phase of the division cycle. Cell-cycle specific substances, such as tubulin inhibitors, were only cytotoxic if the cells were not arrested and the cell cycle was in progress. The assay was performed in 96 well plates. The cell count of induced cells was about three times higher than that of non-induced cells. RKO cells with/without $p27^{Kip1}$ expression (2×10^4 cells /well induced; 6×10^3 cells/well not induced, in 125 μL) were treated with the test compound for 48 h at 37 °C. The controls were untreated cells (±induction). On day 1, the cells were plated (±ponasterone A) and incubated at 37 °C for 24 h. On day 2, the test substance was added (control DMSO) and incubation at 37 °C was continued for another 48 h. Thereafter, a standard XTT assay was carried out.

Flow Cytometric Analysis of Cell-Cycle Status. For a concentration-dependent cell-cycle analysis, K562 cells were exposed to test compounds for 24 h at 37 °C and thereafter collected by centrifugation (3000 rpm, 5 min). The cells were resuspended in 5 mL of phosphate buffered saline (PBS), collected, fixed with ice-cold 70% ethanol (5 mL), and stored at 4 °C for 30 min. Then, the cells were again collected by centrifugation and resuspended in PBS (1.5 mL). DNA content was measured after staining with PI solution (0.2 mg/mL propidium iodide, 2 mg/mL RNase, in PBS) after incubation in the dark for 30 min. Finally, the cells were analyzed with a flow cytometer using an FACS Calibur cytometer system (Becton Dickinson) equipped with an argon-ion laser operated at a wavelength of 488 nm. Analysis was performed with Macintosh Cell Quest software.

For a concentration-dependent cell-cycle analysis, subconfluent KB/HeLa cells were exposed to test compounds for 24 h at 37 °C, detached, and collected. After fixation with 70% ethanol, the DNA was simultaneously stained by propidium iodide and digested with RNase. The DNA content of cells was determined with a FACS CaliburTM cytometer (Beckton Dickinson, Heidelberg, Germany). The number of cells in G2/M phase was calculated by cell-cycle analysis software (Mod Fit LT; VERITY). IC_{50} values were calculated by nonlinear regression (GraphPad Prism).

MTP Isolation and Tubulin Polymerization Assay. Microtubule protein (MTP) consisting of 80−90% tubulin and 10−20%

microtubule associated proteins (MAPs) was isolated from porcine brain by two cycles of temperature-dependent disassembly (0 °C)/reassembly (37 °C), according to the method described by Shelanski et al.[55] Throughout the preparation, a buffer containing 20 mM PIPES (1,4-piperazine diethane sulfonic acid, pH 6.8), 80 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EGTA (ethylene bis (oxyethylenenitrilo) tetraacetic acid), and 1 mM DTT was used. To increase the protein yield, the reassembly steps were performed in the presence of glycerol.[56] The protein concentration was determined by the Lowry procedure using bovine serum albumin as a standard.

The assembly measurements were done directly in cuvettes. To start microtubule formation, the stock MTP solutions were diluted with the preparation buffer to 1.2 mg/mL, GTP was added to 0.6 mM (final concentration), and the samples were transferred into the spectrophotometer (Cary 4E, Varian, Inc.), equipped with a temperature-controlled multichannel cuvette holder, adjusted to 37 °C. Turbidity was recorded over 30 min at 360 nm.[57]

The tubulin effectors were added from stock solutions in DMSO. The final DMSO concentration was 1%. Control measurements were made with DMSO only. To quantify the drug activity, the turbidity signal after 30 min (plateau level, representing the assembly/disassembly steady state) was compared with that of the control samples. The IC_{50} value is defined as the drug concentration that causes a 50% inhibition in relation to the assembly level without the drug.

^{3}H-Colchicine Competition-Binding Assay.[39] ^{3}H-Colchicine was diluted, and biotin-labeled tubulin (T333, Cytoskeleton, Denver, CO) was reconstituted according to the manufacturers protocol. The diluted compounds and the ^{3}H-colchicine were transferred to a 96-well isoplate (PE-Wallac, Boston, MA), and buffer and the reconstituted biotin-labeled tubulin were added. After incubation, streptavidin-coated yttrium SPA beads (Amersham Pharmacia Biotech, Piscataway, NJ) were added, and the bound radioactivity was determined using a MicroBeta Trilux Microplate scintillation counter (PE-Wallac Boston, MA). IC_{50} values were obtained by nonlinear regression (GraphPad Prism).

In Vivo Antitumor Results. Experiments were performed at Unibioscreen SA, Brussels, Belgium. Female CD2F1 mice (Charles River, aged 5−6 weeks) were subcutaneously inoculated with murine P388 tumor fragments of approximately 1 mm^3 at day 0. Treatment was initiated at day 5 after tumor inoculation. Mice were treated intraperitoneally (0.1 mL/10 g body weight) with three doses of **15d** at 100, 50, and 25 mg/kg body weight weekly for three consecutive weeks, for example, on days 5, 12, and 19 after inoculation of the tumor fragments. The controls received intraperitoneal treatment with the vehicle DMSO on days 5, 12, and 19. Body weights of the mice and tumor growth were recorded three times per week. Mice were twice daily observed and sacrificed when moribund. This day of sacrifice is said to be the day of death of the mice.

The effect of treatment on prolongation of survival was evaluated by the % T/C-value and by Kaplan-Meier analysis (log-rank statistics). The % T/C-value is calculated by dividing the day of death of the median mouse in a treated group T by the day of death of the median mouse in the control group C, with the latter said to be 100%. A T/C-value greater than 130% indicates a significant prolongation of survival as compared to that of the vehicle treated group. The second way of evaluating the effect upon survival is by a Kaplan-Meier analysis. The cutoff level for significance by log-rank statistics is set at a p value of $p < 0.05$. Statistical analysis of the effects of treatment on subcutaneous tumor growth was performed with the Mann−Whitney U-test. Here also the statistical cutoff level is $p < 0.05$.

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Supporting Information Available: IR, ^{1}H NMR, and MS data of new compounds **15a**−**15r**, **16a**−**16f**, and **17a**−**17c**. Table of elemental analysis results of all target compounds, cell-cycle analysis data of KB/HeLa cells, effect of treatment with **15d** on subcutaneous tumor growth, and effect of treatment with **15d** on prolongation of survival. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Honore, S.; Pasquier, E.; Braguer, D. Understanding microtubule dynamics for improved cancer therapy. *Cell. Mol. Life Sci.* **2005**, *62*, 3039−3056.
(2) Pellegrini, F.; Budman, D. R. Review: tubulin function, action of antitubulin drugs, and new drug development. *Cancer Invest.* **2005**, *23* (3), 264−273.
(3) Jordan, M. A. Mechanism of action of antitumor drugs that interact with microtubules and tubulin. *Curr. Med. Chem. Anticancer Agents* **2002**, *2* (1), 1−17.
(4) Li, Q.; Sham, H. L. Discovery and development of antimitotic agents that inhibit tubulin polymerisation for the treatment of cancer. *Expert Opin. Ther. Patents* **2002**, *12*, 1663−1702.
(5) Pettit, G. R.; Singh, S. B.; Hamel, E.; Lin, C. M.; Alberts, D. S.; Garcia-Kendall, D. Isolation and structure of the strong cell growth and tubulin inhibitor combretastatin A-4. *Experientia* **1989**, *45* (2), 209−211.
(6) Goodin, S.; Kane, M. P.; Rubin, E. H. Epothilones: mechanism of action and biologic activity. *J. Clin. Oncol.* **2004**, *22* (10), 2015−2025.
(7) Lisowski, V.; Leonce, S.; Kraus-Berthier, L.; Sopkova-de Oliveira Santos, J.; Pierre, A.; Atassi, G.; Caignard, D. H.; Renard, P.; Rault, S. Design, synthesis, and evaluation of novel thienopyrrolizinones as antitubulin agents. *J. Med. Chem.* **2004**, *47* (6), 1448−1464.
(8) Baasner, S.; Emig, P.; Gerlach, M.; Müller, G.; Paulini, K.; Schmidt, P.; Burger, A. M.; Fiebig, H.-H.; Günther, E. G. D-82318 - A novel, synthetic, low molecular weight tubulin inhibitor with potent in vivo antitumor activity. Poster 112. EORTC-NCI-AACR Meeting, Frankfurt, Germany, 2002.
(9) Chen, Z.; Merta, P. J.; Lin, N. H.; Tahir, S. K.; Kovar, P.; Sham, H. L.; Zhang, H. A-432411, a novel indolinone compound that disrupts spindle pole formation and inhibits human cancer cell growth. *Mol. Cancer Ther.* **2005**, *4* (4), 562−568.
(10) Zhang, L. H.; Wu, L.; Raymon, H. K.; Chen, R. S.; Corral, L.; Shirley, M. A.; Narla, R. K.; Gamez, J.; Muller, G. W.; Stirling, D. I.; Bartlett, J. B.; Schafer, P. H.; Payvandi, F. The synthetic compound CC-5079 is a potent inhibitor of tubulin polymerization and tumor necrosis factor-alpha production with antitumor activity. *Cancer Res.* **2006**, *66* (2), 951−959.
(11) Prinz, H.; Ishii, Y.; Hirano, T.; Stoiber, T.; Camacho Gomez, J. A.; Schmidt, P.; Düssmann, H.; Burger, A. M.; Prehn, J. H.; Günther, E. G.; Unger, E.; Umezawa, K. Novel benzylidene-9(10*H*)-anthracenones as highly active antimicrotubule agents. Synthesis, antiproliferative activity, and inhibition of tubulin polymerization. *J. Med. Chem.* **2003**, *46* (15), 3382−3394.
(12) Lima, L. M.; Barreiro, E. J. Bioisosterism: a useful strategy for molecular modification and drug design. *Curr. Med. Chem.* **2005**, *12* (1), 23−49.
(13) Flynn, B. L.; Flynn, G. P.; Hamel, E.; Jung, M. K. The synthesis and tubulin binding activity of thiophene-based analogues of combretastatin A-4. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2341−2343.
(14) Pinney, K. G.; Bounds, A. D.; Dingeman, K. M.; Mocharla, V. P.; Pettit, G. R.; Bai, R.; Hamel, E. A new anti-tubulin agent containing the benzo[*b*]thiophene ring system. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1081−1086.
(15) MacDowell, D. W. H.; Wisowaty, J. C. Keto-enol tautomerism in the thiophene analogs of anthrone. II. Benzodithiophenes. *J. Org. Chem.* **1971**, *36*, 4004−4012.
(16) Peters, A. T.; Walker, D. Intermediates and dyes. Part V. Derivatives of thiophanthrene-4:9-quinone. *J. Chem. Soc.* **1957**, 1525−1532.
(17) Weinmayr, V. *o*-(2-Thenoyl)-benzoic acids and thiophanthraquinones. *J. Am. Chem. Soc.* **1952**, *74*, 4353−4357.
(18) Zani, C. L.; Chiari, E.; Krettli, A. U.; Murta, S. M.; Cunningham, M. L.; Fairlamb, A. H.; Romanha, A. J. Anti-plasmodial and anti-trypanosomal activity of synthetic naphtho[2,3-*b*]thiophene-4,9-quinones. *Bioorg. Med Chem.* **1997**, *5* (12), 2185−2192.
(19) Schroeder, H. E.; Weinmayr, V. The synthesis of thiophanthra quinones from thenoyl and thenylbenzoic acids. *J. Am. Chem. Soc.* **1952**, *74*, 4357−4361.
(20) MacDowell, D. W. H.; Wisowaty, J. C. Keto-enol tautomerism in the thiophene analogs of anthrone. I. Derivatives of naphtho[2,3-*b*]-thiophene and naphtho[2,3-*c*]thiophene. *J. Org. Chem.* **1971**, *36*, 3999−4004.
(21) El-Shafie, S. M. M. Synthesis of 10-arylidene-9-anthrones and related compounds. *Indian J. Chem.* **1978**, *16B*, 828−830.

(22) Bergmann, E. D.; Rabinovitz, M.; Glily, S. A ring enlargement in the 9,10-dihydroanthracene series. *Tetrahedron Suppl.* **1966**, *8*, 141−148.
(23) Weitz, E. Über einige Anthronabkömmlinge. *Liebigs Ann. Chem.* **1919**, *418*, 29−35.
(24) Rappoport, Z.; Apeloig, Y.; Greenblatt, J. Vinylic cations from solvolysis. 29. Solvolysis of 9-(α-bromoarylidene)anthrones as a probe to the reactivity−selectivity relationship in solvolysis reactions. *J. Am. Chem. Soc.* **1980**, *102*, 3837−3848.
(25) Müller, K.; Gürster, D.; Piwek, S.; Wiegrebe, W. Antipsoriatic anthrones with modulated redox properties. 1. Novel 10-substituted 1,8-dihydroxy-9(10*H*)-anthracenones as inhibitors of 5-lipoxygenase. *J. Med. Chem.* **1993**, *36* (25), 4099−4107.
(26) Dimmel, D. R.; Shepard, D. Regioselective alkylation of anthrahydroquinone and anthrone in water with quinonemethides and other alkylating agents. *J. Org. Chem.* **1982**, *47*, 22−29.
(27) Lozzio, C. B.; Lozzio, B. B. Human chronic myelogenous leukemia cell-line with positive philadelphia chromosome. *Blood* **1975**, *45*, 321−334.
(28) Lawrence, N. J.; Rennison, D.; Woo, M.; McGown, A. T.; Hadfield, J. A. Antimitotic and cell growth inhibitory properties of combretastatin A-4-like ethers. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 51−54.
(29) Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. Evaluation of a soluble tetrazolium/formazane assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.* **1988**, *48*, 4827−4833.
(30) Schmidt, M.; Lu, Y.; Parant, J. M.; Lozano, G.; Bacher, G.; Beckers, T.; Fan, Z. Differential roles of $p21^{Waf1}$ and $p27^{Kip1}$ in modulating chemosensitivity and their possible application in drug discovery studies. *Mol. Pharmacol.* **2001**, *60*, 900−906.
(31) Dumontet, C.; Jaffrezou, J. P.; Tsuchiya, E.; Duran, G. E.; Chen, G.; Derry, W. B.; Wilson, L.; Jordan, M. A.; Sikic, B. I. Resistance to microtubule-targeted cytotoxins in a K562 leukemia cell variant associated with altered tubulin expression and polymerization. *Bull. Cancer* **2004**, *91* (5), 81−112.
(32) Dumontet, C.; Sikic, B. I. Mechanism of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. *J. Clin. Oncol.* **1999**, *3*, 1061−1070.
(33) Fardel, O.; Lecureur, V.; Guillouzo, A. The P-glycoprotein multidrug transporter. *Gen. Pharmacol.* **1996**, *27*, 1283−1291.
(34) Cole, S. P.; Deeley, R. G. Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP. *Bioessays* **1998**, *20*, 931−940.
(35) Verrills, N. M.; Kavallaris, M. Improving the targeting of tubulin-binding agents: lessons from drug resistance studies. *Curr. Pharm. Des.* **2005**, *11* (13), 1719−1733.
(36) De Martino, G.; Edler, M. C.; La Regina, G.; Coluccia, A.; Barbera, M. C.; Barrow, D.; Nicholson, R. I.; Chiosis, G.; Brancale, A.; Hamel, E.; Artico, M.; Silvestri, R. New arylthioindoles: potent inhibitors of tubulin polymerization. 2. Structure−activity relationships and molecular modeling studies. *J. Med. Chem.* **2006**, *49* (3), 947−954.
(37) Kuo, C. C.; Hsieh, H. P.; Pan, W. Y.; Chen, C. P.; Liou, J. P.; Lee, S. J.; Chang, Y. L.; Chen, L. T.; Chen, C. T.; Chang, J. Y. BPR0L075, a novel synthetic indole compound with antimitotic activity in human cancer cells, exerts effective antitumoral activity in vivo. *Cancer Res.* **2004**, *64* (13), 4621−4628.
(38) Mahboobi, S.; Pongratz, H.; Hufsky, H.; Hockemeyer, J.; Frieser, M.; Lyssenko, A.; Paper, D. H.; Burgermeister, J.; Bohmer, F. D.; Fiebig, H. H.; Burger, A. M.; Baasner, S.; Beckers, T. Synthetic 2-aroylindole derivatives as a new class of potent tubulin-inhibitory, antimitotic agents. *J. Med. Chem.* **2001**, *44* (26), 4535−4553.
(39) Tahir, S. K.; Kovar, P.; Rosenberg, S.; Ng, S.-C. A rapid colchicine competition-binding scintillation proximity assay using biotin-labeled colchicine. *Biotechniques* **2000**, *29*, 156−160.
(40) Bai, R.; Roach, M. C.; Jayaram, S. K.; Barkoczy, J.; Pettit, G. R.; Luduena, R. F.; Hamel, E. Differential effects of active isomers, segments, and analogs of dolastatin 10 on ligand interactions with tubulin. Correlation with cytotoxicity. *Biochem. Pharmacol.* **1993**, *45*, 1503−1515.
(41) Bai, R. L.; Pettit, G. R.; Hamel, E. Binding of dolastatin 10 to tubulin at a distinct site for peptide antimitotic agents near the exchangeable nucleotide and vinca alkaloid sites. *J. Biol. Chem.* **1990**, *265*, 17141−17149.
(42) Carruthers, W.; Douglas, A. G.; Hill, J. Synthesis of naphtho[2,3-*b*]thiophen. *J. Chem. Soc.* **1962**, 704−708.
(43) Müller, K.; Breu, K. 10-omega-phenylalkyl-9(10*H*)-anthracenones as inhibitors of keratinocyte growth with reduced membrane damaging properties. *Bioorg. Med. Chem. Lett.* **1998**, *8* (22), 3211−3216.
(44) Schaltegger, A.; Steiger, W. Darstellung von 10-Alkylderivaten des 1,8-Dihydroxy-9(10*H*)-anthracenons (Dithranol). *Arch. Pharm.* **1986**, *319*, 575−576.
(45) Venkateswarlu, S.; Raju, M. S.; Subbaraju, G. V. Synthesis and biological activity of isoamoenylin, a metabolite of *Dendrobium amoenum*. *Biosci. Biotechnol. Biochem.* **2002**, *66* (10), 2236−2238.
(46) Figadère, B.; Chaboche, C.; Franck, X.; Peyrat, J.-F.; Cavé, A. Carbonyl reduction and functionalized aldehydes and ketones by tri-*n*-butyltin hydride and SiO_2. *J. Org. Chem.* **1994**, *59*, 7138−7141.
(47) Kröhnke, F.; Schmeiss, H.; Gottstein, W. Über Enolbetaine, VIII. Mitteil.: Umsetzungen mit Benzyl- und Cinnamoylhalogeniden zu Phenäthyl- und Homocinnamoyl-pyridiniumsalzen. *Chem. Ber.* **1951**, *84*, 131−138.
(48) Guziec F. S., Jr.; Wei, D. Convenient halodeamination and hydrodeamination of primary amines. *J. Org. Chem.* **1992**, *57*, 3772−3776.
(49) Rychener, M.; Steiger, W.; Krebs, A. [Senecioyldithranol and beta-carbethoxypropionyldithranol: synthesis and glucose-6-phosphate dehydrogenase inhibition of two new 10-acyl derivatives of the antipsoriatic, dithranol]. *Pharm. Acta Helv.* **1988**, *63*, 111−116.
(50) Amedio J. C., Jr.; Sunay, U. B.; Repic, O. Metalated aromatic carboxylic acids: improved synthesis of SDZ HUL412. *Synth. Commun.* **1995**, *25*, 667−680.
(51) Biel, J. H.; Drukker, A. E.; Mitchell, T. F.; Sprengeler, E. P.; Nuhfer, P. A.; Conway, A. C.; Horita, A. Central stimulants. Chemistry and structure−activity relationships of aralkyl hydrazines. *J. Am. Chem. Soc.* **1959**, *81*, 2805−2813.
(52) Kruse, L. I.; Kaiser, C.; DeWolf, W. E., Jr.; Frazee, J. S.; Garvey, E.; Hilbert, E. L.; Faulkner, W. A.; Flaim, K. E.; Sawyer, J. L.; Berkowitz, B. A. Multisubstrate inhibitors of dopamine beta-hydroxylase. 1. Some 1-phenyl and 1-phenyl-bridged derivatives of imidazole-2-thione. *J. Med. Chem.* **1986**, *29* (12), 2465−2472.
(53) Schmidt, M.; Lu, Y.; Liu, B.; Fang, M.; Mendelsohn, J.; Fan, Z. Differential modulation of paclitaxel-mediated apoptosis by p21waf1 and p27kip1. *Oncogene* **2000**, *19*, 2423−2429.
(54) Bacher, G.; Nickel, B.; Emig, P.; Vanhoefer, U.; Seeber, S.; Shandra, A.; Klenner, T.; Beckers, T. D-24851, a novel synthetic microtubule inhibitor, exerts curative antitumoral activity in vivo, shows efficiacy toward multidrug-resistant tumor cells, and lacks neurotoxicity. *Cancer Res.* **2001**, *61*, 392−399.
(55) Shelanski, M. L.; Gaskin, F.; Cantor, C. R. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 765−768.
(56) Vater, W.; Böhm, K. J.; Unger, E. A simple method to obtain brain microtubule protein poor in microtubule-associated proteins. *Acta Histochem. Suppl.* **1986**, *33*, 123−129.
(57) Gaskin, F.; Cantor, C. R.; Shelanski, M. L. Turbidimetric studies of the in vitro assembly and disassembly of porcine neurotubules. *J. Mol. Biol.* **1974**, *89* (4), 737−755.

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