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Polyalkoxy Substituted 4*H*-Chromenes: Synthesis by Domino Reaction and Anticancer Activity

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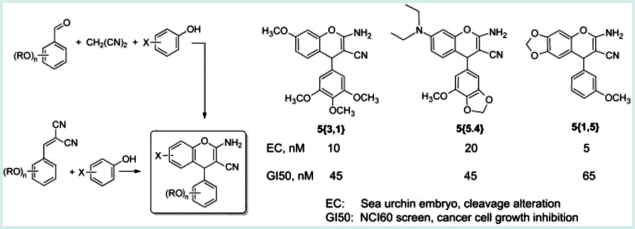
Supporting Information

ABSTRACT: A series of 4*H*-chromenes containing various modifications in the ring B and polyalkoxy substituents in the ring E has been synthesized by Knoevenagel−Michael−hetero-Thorpe−Ziegler three-component domino reaction with the overall yield of 45−82%. The targeted molecules were evaluated in a phenotypic sea urchin embryo assay for antimitotic and microtubule destabilizing activity. The most active compounds **5**{*1,5*} and **5**{*5,5*} featured sesamol-derived ring B and *m*-methoxyphenyl or *m*-methoxymethylenedioxyphenyl ring E. Compounds **5**{*3,1*}, **5**{*1,2*}, **5**{*5,4*}, **5**{*1,5*}, and **5**{*5,5*} exhibited strong cytotoxicity in the NCI60 human tumor cell line anticancer drug screen. Surprisingly, cell growth inhibition caused by these agents was more pronounced in the multidrug resistant NCI/ADR-RES cells than the parent OVCAR-8 cell line. The results suggest that polyalkoxy substited 4*H*-chromenes may prove to be advantageous for further design as anticancer agents.



KEYWORDS: *4H-chromenes, domino reaction, microtubule destabilizing agents, sea urchin embryo, cytotoxicity*

INTRODUCTION

Microtubules are highly dynamic cytoskeleton organelles that play an essential role in cell division. At mitosis they rearrange to form mitotic spindle responsible for proper orientation and segregation of chromosomes into daughter cells. Hence, targeting tubulin in rapidly dividing tumor cells is a sound strategy for anticancer therapy. A number of natural products and their synthetic derivatives exhibit antiproliferative activity by interfering with tubulin polymerization and depolymerization resulting in mitotic arrest.[1] However, the development of multidrug resistance and high general toxicity limit their use in oncology. Therefore, there is ongoing need for small molecule tubulin inhibitors that display better therapeutic window and overcome multidrug resistance.

4-Aryl-4*H*-chromenes (Figure 1) were found to exhibit strong cytotoxicity against human cancer cells involving microtubule depolymerization, G2/M cell cycle arrest, caspase-dependent apoptotic cell death, and tumor vasculature disruption.[2−10] Compound EPC2407 (Figure 1) is currently in phase I/II clinical trials as vascular targeting anticancer agent and apoptosis inducer for the treatment of patients with advanced solid tumors.[10] 4-Aryl-4*H*-chromenes were identified as microtubule destabilizers with binding site at or close to the colchicine binding site.[2] They may be considered as synthetically feasible structural analogues of natural antimitotic lignan podophyllotoxin (PT), which is the strong microtubule destabilizing agent that binds to the colchicine site of tubulin.[11] Although the use of PT as anticancer agent was unsuccessful due to its strong systemic toxicity, several semisynthetic PT derivatives, including etoposide (Figure 1), teniposide, and etopophos, are currently in clinical use for the treatment of

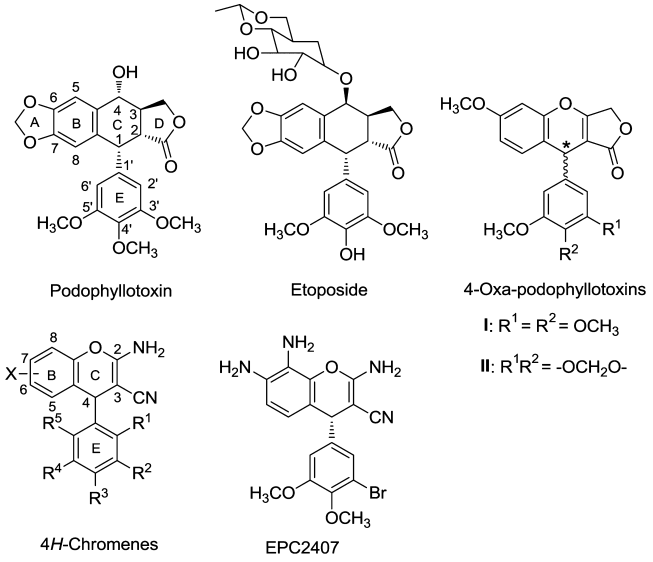


Figure 1. Semisynthetic podophyllotoxin derivatives.

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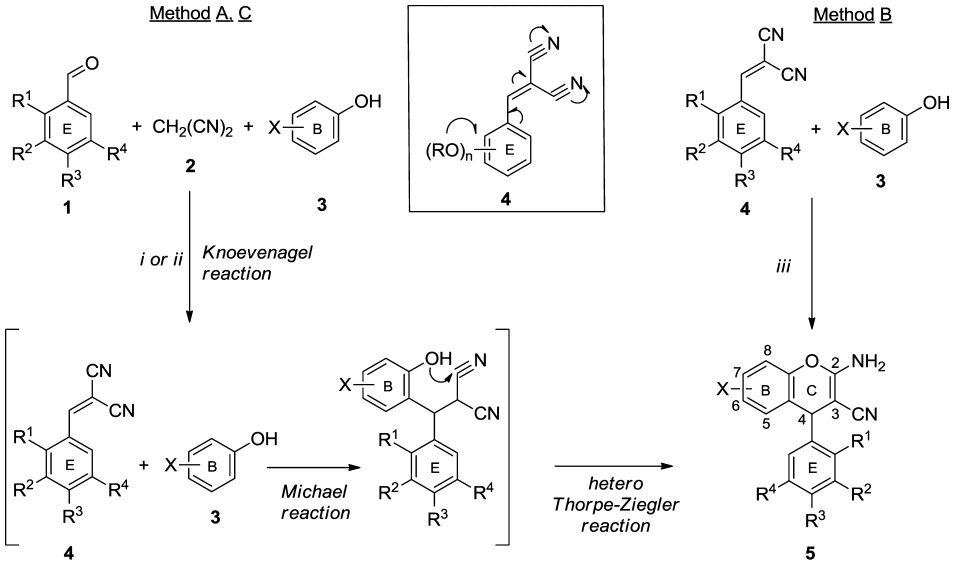
Scheme 1. Synthesis of 2-Amino-4*H*-chromenes 5[a]



[a]Reagents and conditions: (i) EtOH, Et_3N, reflux, 5−30 min (method A); (ii) EtOH, Et_3N, MW 300 W, 4−5 min (method C); (iii) EtOH, Et_3N, reflux, 5−30 min (method B). Insertion: Electronic effects in polyalkoxybenzylidene malononitriles **4**.

various malignancies. These compounds exhibit a mechanism of action entirely different from that of the parent PT, involving strong DNA-topoisomerase II inhibition, S−G2 cell cycle arrest, and subsequent apopotosis.[12]

The stereo structure of PT molecule with four chiral centers (Figure 1) was reported to be crucial for antitumor activity,[13] which makes challenging the structure−activity relationship (SAR) studies. Numerous attempts have been made to obtain potent synthetically feasible analogues of PT with a heteroatom in the ring C to prevent epimerization. Namely, 4-oxa-PT derivatives featured a single chiral center (**I**, **II**; Figure 1), displayed strong antimitotic and microtubule destabilizing effects, as well as high cytotoxicity against a panel of human cancer cell lines.[14] Unfortunately, the synthetic approach for 6-methoxy-4-oxa-PT-derivatives **I** and **II** resulted in a low overall yield (3−6%) due to ambiguous reactivity of tetronic acid in Mannich reaction.[14,15]

Considering synthetic feasibility, we aimed at designing a simple procedure to prepare 4-aryl-4*H*-chromenes that contain cyano and amino groups instead of the lactone ring D of PT (Figure 1), as they could be readily accessible while maintaining antimitotic potency. The axial orientation of the ring E in PT molecule was proposed to restrict flexibility at the C_1 center and subsequently enhance antiproliferative effects.[13,16] Therefore, a modification of the ring E by different polyalkoxy substituents may further hamper its spatial rotation, thus increasing antimitotic activity of the targeted compounds. In addition, small hydrophobic moieties at C_7 in the ring B of 4*H*-chromenes, such as methoxy, amino, dimethylamino, and ethylamino groups, were found to be beneficial for cancer cell growth inhibition and caspase-dependent apoptosis.[4] Accordingly, in the present study 4-polyalkoxyaryl-4*H*-chromenes with the ring B substitued by methoxy, methylenedioxy, and amino groups have been synthesized.

RESULTS AND DISCUSSION

Chemistry. Previously we have designed the three-component synthesis of 2-amino-4*H*-pyrans and 2-amino-4*H*-chromenes.[17,18] Herein, we reported the preparation of 4-aryl-4*H*-chromenes via the three-component domino reaction of polyalkoxybenzaldehydes **1**, malononitrile **2**, and phenols **3**. It should be noted that in polyalkoxybenzylidene malononitriles **4** the electronic effects of the substituents (Scheme 1, Insertion) hampered activation of the C═C double bond, requiring rather harsh conditions to undergo Michael reaction. Therefore the domino reaction was carried out by heating in the microwave field, affording the yield increase by 8−12%.

Three-component reaction of aldehydes **1**, malononitrile **2**, and 3-phenols **3** (Method A, Scheme 1) was carried out by heating in ethanol in the presence of catalytic amounts of triethylamine. Under these conditions substituted 4-aryl-4*H*-chromenes **5** were obtained in acceptable yields (45−82%). Most probably, formation of the targeted products **5** proceeded

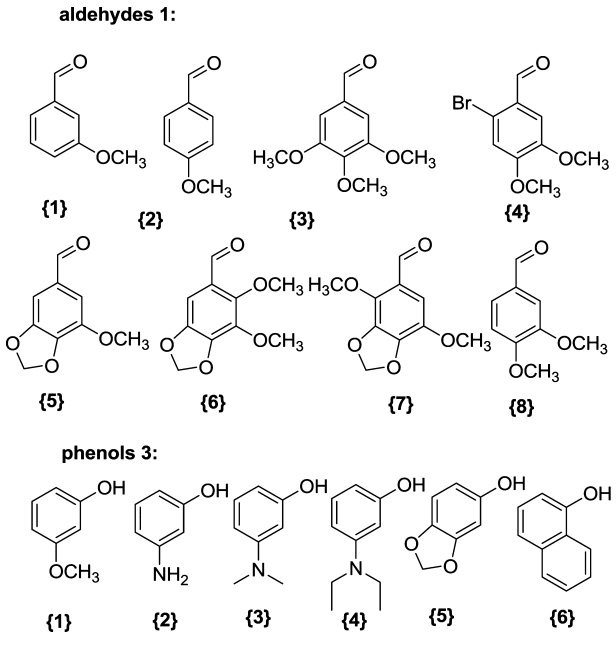


Figure 2. Diversity of reagents.

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Table 1. 2-Amino-4*H*-chromenes 5

entry	product	X	R_1	R_2	R_3	R_4	yield (%) method A	yield (%) method B	yield (%) method C
1	5{3,1}	7-OCH_3	H	OCH_3	OCH_3	OCH_3	12		
2	5{3,2}	7-NH_2	H	OCH_3	OCH_3	OCH_3	53		
3	5{4,2}	7-NH_2	Br	H	OCH_3	OCH_3	45	52	53
4	5{2,2}	7-NH_2	H	H	OCH_3	H	82		
5	5{1,2}	7-NH_2	H	OCH_3	H	H	74		
6	5{4,3}	7-$N(CH_3)_2$	Br	H	OCH_3	OCH_3	52		
7	5{7,3}	7-$N(CH_3)_2$	OCH_3	$-OCH_2O-$	$-OCH_2O-$	OCH_3	62	70	74
8	5{6,3}	7-$N(CH_3)_2$	OCH_3	OCH_3	$-OCH_2O-$	$-OCH_2O-$	70	77	79
9	5{8,4}	7-$N(C_2H_5)_2$	H	OCH_3	OCH_3	H	75		
10	5{5,4}	7-$N(C_2H_5)_2$	H	OCH_3	-OCH_2O-	-OCH_2O-	57	65	
11	5{1,5}	6,7-OCH_2O-	H	OCH_3	H	H	82		
12	5{5,5}	6,7-OCH_2O-	H	OCH_3	$-OCH_2O-$	$-OCH_2O-$	53		
13	5{4,6}	7,8-C_6H_4	Br	H	OCH_3	OCH_3	56		

as domino reaction including Knoevenagel reaction, Michael reaction, and hetero-Thorpe–Ziegler reaction. Generally, domino reactions are widely used in the synthesis of a variety of organic compounds, including natural products.[19] However, this particular sequence of reactions has not been previously classified as domino. The introduction of unsaturated nitrile **4**, obtained previously from aldehyde **1** and malononitrile **2**, into the reaction provided the indirect evidence of the domino mechanism of the compounds interaction (Method B, Scheme 1). Noteworthy, the use of 3-methoxy-4.5-methylenedioxybenzonitrile (**4**, $R_2 = OCH_3$, $R_3–R_4 = -OCH_2O-$) as the chemical precursor (Method B) increased the yield of the targeted compound 5{5,4} by ~8% comparing to the yield of the same compound in Method A. However, overall yield of the products in Method B was comparable to that in Method A because the preliminary synthesis of nitriles **4** by Knoevenagel reaction proceeded with restricted yield. Application of the microwave irradiation[18] increased the yield by 8–12% (Method C), simultaneously enhancing the amount of tarry byproduct. Hence, crystallization of products **5** in the presence of activated charcoal was required.

Structures of the synthesized compounds were confirmed by spectral analyses (See Supporting Information). In IR spectra of compounds **5** characteristic band of conjugated nitrile group (2184–2197 cm^{-1}), as well as stretching bands of NH_2 group (3113–3480 cm^{-1}), bending bands of NH_2 (1605–1664 cm^{-1}) have been observed. The ^1H NMR spectra of the title compounds featured C(4)H proton singlet in the region 4.45–5.32 ppm and broad singlet of exchangeable NH_2 group (6.36–7.14 ppm). Signals of the corresponding aromatic and aliphatic protons were present as well. Mass-spectra of all aminochromenes **5** contained highly intensive peak of molecular ion.

Biological Evaluation: SAR Studies. All synthesized 4*H*-chromenes were further evaluated in a phenotypic sea urchin embryo assay[20] for their antimitotic and microtubule destabilizing activity using PT as a benchmark reference compound (Table 2). The assay includes (i) fertilized egg test for antimitotic activity displayed by cleavage alteration/arrest, and (ii) behavioral monitoring of a free-swimming blastulae treated immediately after hatching. The lack of forward movement, settlement to the bottom of the culture vessel, and rapid spinning of embryos around the animal–vegetal axis suggests a microtubule destabilizing activity caused by a molecule (video illustrations are available at http://www.chemblock.com).

Table 2. Effects of 2-Amino-4*H*-chromenes on the Sea Urchin Embryos and Human Cancer Cells

compound	EC (μM)[a] cleavage alteration	EC (μM)[a] cleavage arrest	EC (μM)[a] embryo spinning	cell growth inhibition (average GI_{50}, μM)[b]
PT	0.02	0.05	0.5	
I[c]	0.005	0.05	0.2	0.631
II[c]	0.002	0.01	0.1	
5{3,1}	0.01	0.05	0.5	0.045
5{3,2}	1	4	>5	
5{4,2}	2	>2	>2	
5{2,2}	2	>4	>4	
5{1,2}	0.1	1	5	0.257
5{4,3}	0.5	2	5	
5{7,3}	1	4	>4	
5{6,3}	0.5	2	4	
5{8,4}	0.1	0.5	4	
5{5,4}	0.02	0.05	0.2	0.045
5{1,5}	0.005	0.05	0.2	0.065
5{1,5}-1	0.1	1	5	
5{1,5}-2	0.002	0.02	0.1	
5{5,5}	0.005	0.05	0.2	0.389
5{4,6}	0.2	4	5	

[a]The sea urchin embryo assay was conducted as described in ref 20. Fertilized eggs and hatched blastulae were exposed to 2-fold decreasing concentrations of compounds. Duplicate measurements showed no differences in effective threshold concentration (EC) values. [b]NCI60 anticancer drug screen; GI_{50}: Concentration required for 50% cell growth inhibition. [c]Data from ref 14.

As evidenced from Table 2, 4-aryl-4*H*-chromenes 5{3,1}, 5{1,2}, 5{4,3}, 5{6,3}, 5{8,4}, 5{5,4}, 5{1,5}, 5{5,5}, and 5{4,6} caused noticeable cleavage alteration, cleavage arrest, and embryo spinning, suggesting their antimitotic microtubule destabilizing activity. Less potent compounds 5{3,2} and 5{7,3} could be considered as tubulin destabilizers as well, although they did not cause embryo spinning, since these compounds induced formation of tuberculate eggs typical of tubulin destabilizers.[20,21]

Recent SAR studies of oxa-PT analogues showed that 6-methoxy moiety in the ring B in combination with the myristicin-derived or 2,3,4-trimethoxyphenyl ring E resulted in potent cytotoxic tubulin destabilizing compounds (**I**, **II**; Figure

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1, Table 2).[14] Similarly, chromene 5{3,1}, with cyano and amino groups instead of the lactone ring D, displayed high antimitotic microtubule destabilizing activity in the sea urchin embryo assay comparable with the effect of the parent PT. Previously this compound has been identified as strong proapoptotic agent in the caspase activation assay.[4] Replacement of 7-methoxy moiety by 7-amino group resulted in a marked reduction of activity and inability to induce embryo spinning (Table 2, 5{3,1} vs 5{3,2}). Chromene 5{1,2} featuring 3-methoxybenzene ring E was 10 times more active than 5{3,2}, whereas 5{2,2} containing *p*-methoxy substituted ring E exhibited only moderate cleavage alteration effect. It should be reported previously that the replacement of 3-methoxy group in the E-ring with bromine enhanced the potency in caspase activation assay and inhibited cancer cell growth in submicromolar concentration range.[3,4,10a] Unfortunately, in our assay we were unable to test compound 5{4,2} with 2-bromine in the ring E due to its limited solubility in DMSO and the presence of microscopic crystals in 5−10 mM stock solutions. Notably, the respective analogue 5{4,3} with 7-dimethylamino substituent in the ring B showed rather low antiproliferative antitubulin effect similar to the activity of 5{6,3} with 2,3-dimethoxy-4,5-methylenedioxybenzene ring E. The corresponding isomer 5{7,3} containing 2,5-dimethoxy-3,4-methylenedioxybenzene was less potent. Conjugation of phenyl ring to C_7C_8 positions of the ring B instead of 7-dimethylamino group did not influence the activity of Br-substituted chromenes (Table 2, 5{4,3} and 5{4,6}). For compounds featuring 7-diethylamino group, the 3-methoxy-4,5-methylenedioxybenzene analogue 5{5,4} exhibited high activity in the sea urchin embryo assay, whereas 5{8,4} with 3,4-dimethoxyphenyl ring E was less potent. Conjugation of methylenedioxy functionality to C_6C_7 positions of the ring B markedly increased antimitotic activity of 4-aryl-4*H*-chromenes, yielding the most potent compounds 5{1,5} and 5{5,5} with EC values of 5 nM (Table 2). In this case both 3-methoxyphenyl and 3-methoxy-4,5-methylenedioxybenzene ring E afforded the same antimitotic potency. Chromenes 5{1,5} and 5{5,5} closely resembled PT in the A−B-C ring assembly. These molecules were found to be more potent than PT in the sea urchin embryo assay. We suggested that the replacement of the lactone ring in PT by cyano and amino groups could be beneficial for antimitotic microtubule destabilizing activity.

The stereo structure of PT-derived microtubule destabilizers was reported to contribute significantly to their activity,[13] For 4-aza-PTs, only *R*-enantiomers displayed pronounced cytotoxicity against cultured insect cells and larvae[15] as well as against HeLa and MCF-7 human cancer cells.[22] In our previous studies *R*-isomer of 4-aza-PT was found to cause cleavage alteration and cleavage arrest of the sea urchin embryo at 5 and 50 nM, respectively, whereas the corresponding *S*-isomer was less active exhibiting cleavage abnormalities at 50 nM and cleavage arrest at 200 nM.[23] Similarly, R-isomer of substituted 4*H*-chromene (EPC2407, Figure 1, 3) was shown to activate caspases, induce apoptosis in human cancer cells, and exhibit vascular disruption effect in nanomolar concentration range, whereas the caspase activation potency of corresponding S-isomer was ∼50−100 times less.[10,24]

Herein, we studied the effect of stereochemistry on the antimitotic antitubulin activity of compound 5{1,5} in the sea urchin embryo assay. A racemic 5{1,5} was separated by chiral HPLC to yield pure enantiomers (Figure 3; Figure S1, Supporting Information). The isomer 5{1,5}-2 exhibited high antimitotic effect, inducing cleavage alteration and cleavage arrest at concentration of 2 and 20 nM, respectively, which was twice more than the activity of racemic 5{1,5} (Table 2). The activity of isomer 5{1,5}-1 was 50 times less (EC = 0.1 and 1 μM, respectively). Both enantiomers triggered embryo spinning, suggesting their microtubule destabilizing properties. The absolute configuration of these chiral isomers is in progress.

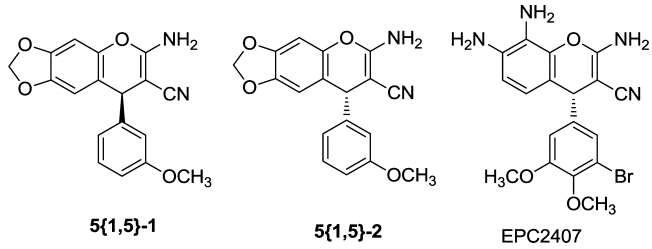

Figure 3. Structures of 5{1,5} enantiomers and compound EPC2407.[10,24] The spatial structures of 5{1,5} are presented by analogy with EPC2407.

Chromenes 5{3,1}, 5{1,2}, 5{5,4}, 5{1,5}, and 5{5,5}, determined as strong antimitotics in the sea urchin embryo assay, were further selected for the NCI60 anticancer drug screen. These compounds displayed high cytotoxicity against a panel of human cancer cell lines with GI_{50} values in the nanomolar concentration range (Table 2; Table S1, Supporting Information). Leukemia cells (K-562 and SR), nonsmall cell lung carcinoma (NCI-H522), CNS carcinoma (SF-295), melanomas (MDA-MB-435 and UACC-62), and ovarian carcinoma (NCI/ADR-RES) were the most sensitive cell lines to 5{5,4} with GI_{50} < 10 nM. Importantly, the replacement of the lactone ring by cyano and amino groups in compounds with 2,3,4-trimethoxyphenyl ring E increased cytotoxicity (5{3,1} vs **I**; Table 2; Table S1, Supporting Information). Generally, the sensitivity of sea urchin embryos to chromenes was somewhat higher than that of cancer cells, probably due to the differences between mitotic spindle microtubules in frequently dividing sea urchin blastomeres vs predominantly interphase microtubules in cultured cancer cells altered by these tubulin targeting agents.

The multidrug resistant ovarian cancer cell line NCI/ADR-RES has been proved to derive from ovarian cancer cell line OVCAR-8 (http://dtp.nci.nih.gov/docs/misc/common_files/NCI-ADRres.html). This line overexpresses P-glycoprotein responsible for the transport of various substrates across cell membrane, resulting in the type I multidrug resistance phenotype. Importantly, all tested 4-aryl-4*H*-chromenes exhibited higher cytotoxicity against NCI/ADR-RES cells than against the parent OVCAR-8 cells (Table 3), suggesting their potential to overcome multidrug resistance.

CONCLUSION

In summary, a novel synthetic approach has been developed to obtain polyalkoxy substituted 4-aryl-4*H*-chromenes, namely, a three-component domino reaction of the respective polyalkoxybenzaldehydes, malononitrile, and amino or alkoxyphenols. The targeted compounds were evaluated using the in vivo phenotypic sea urchin embryo assay that allowed for rapid and reproducible identification of antitubulin molecules. A variety of modifications in the ring B and 4-aryl fragment yielded potent antimitotic agents with microtubule destabilizing activity. The

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Table 3. Growth Inhibition of OVCAR-8 and NCI/ADR-RES Cell Lines

compound	cell growth inhibition (GI_{50}, nM)[a]	
	OVCAR-8[b]	NCI/ADR-RES[c]
5{3,1}	34.5	19.9
5{1,2}	701.0	240.0
5{5,4}	36.8	<10
5{1,5}	97.1	30.2
5{5,5}	435.0	101.0

[a]GI_{50}: Concentration required for 50% cell growth inhibition. [b]OVCAR-8: ovarian cancer cell line 8. [c]NCI/ADR-RES: P-glycoprotein-overexpressing multidrug resistant cell line derived from OVCAR-8.

most active chromenes with EC values of 5 nM featured sesamol-derived ring B, closely resembling the structure of PT. Compounds **5**{*3,1*}, **5**{*1,2*}, **5**{*5,4*}, **5**{*1,5*}, and **5**{*5,5*} markedly inhibited human cancer cell growth in the NCI60 cytotoxicity screen. Interestingly, the multidrug resistant NCI/ADR-RES cells showed higher sensitivity to these agents than the parent OVCAR-8 cell line. The results suggest that synthetically feasible polyalkoxy substited 4*H*-chromenes may prove to be advantageous for further design as anticancer agents.

EXPERIMENTAL PROCEDURES

Elemental microanalyses were obtained on an Perkin-Elmer 2400 CHN analyzer. Mass spectra were collected on the Varian MAT-CH-6 spectrometer with direct sample injection at an ionization voltage of 70 eV. IR spectra were recorded on IFS-113v Bruker in KBr pellets (1:200); the frequencies were expressed in cm^{-1}. The ^1H NMR spectra were recorded on Bruker DRX-500 (500 MHz) and Bruker AM-300 (300 MHz) using internal standard with DMSO-D6 as the solvent; the chemical shifts were reported in ppm (δ) and coupling constants (J) values were given in Hertz (Hz). Melting points were measured on a Kofler bench. Completion of the reactions and purity of the obtained products were monitored by thin layer chromatography on the Silufol UV-254 plates using hexane-acetone mixture (5:3) as an eluent and iodine vapor as a stain.

Synthesis of 2-Amino-4*H*-chromenes 5. *General Procedures. Method A.* Aromatic aldehyde **1** (1 mmol), malononitrile **2** (1 mmol), and the appropriate hydroxyaromatic compound **3** (1 mmol) were dissolved in 5 mL of EtOH, one drop of Et_3N was added and the reaction mixture was refluxed for 5−30 min (TLC control). After cooling of the reaction mixture to 4 °C for 12 h fine crystalline precipitate was formed, which was filtered off, washed successively with ethanol (3 mL) and hexanes (3 mL) and dried.

Method B ***5****({4,2}, {7,3}, {6,3}, {5,4})*. To a mixture of one of the hydroxyaromatic compounds **3** (1 mmol) and arylidene malononitrile **4** (1 mmol), which had been preliminary synthesized from the corresponding aldehyde **1** and malononitrile **2**, EtOH (5 mL) one drop of Et3N was added and the reaction mixture was refluxed for 5−30 min (TLC control). After cooling of the reaction mixture to 4 °C for 12 h fine crystalline precipitate was formed, which was filtered off, washed successively with ethanol (3 mL) and hexanes (3 mL) and dried.

Method C ***5****({4,2}, {7,3}, {6,3})*. Aromatic aldehyde **1** (1 mmol), malononitrile **2** (1 mmol), hydroxyaromatic compound **3** (1 mmol), 5 mL of EtOH and one drop of Et_3N were mixed together in a 10 mL Pyrex glass sample holder (CEM). The reaction was carried out in a closed vessel using a focused microwave synthesis system (CEM Discover BenchMate) under continuous stirring. The incubation time was 4−5 min with a fixed microwave irradiation power at 300 W and a maximum temperature at 70 °C (TLC control). The solid formed after cooling of the reaction mixture to 4 °C for 12 h was recrystallized from EtOH in the presence of charcoal, filtered off, washed successively with ethanol (3 mL) and hexanes (3 mL) and dried.

Biology: Materials and Methods. *Sea Urchin Embryo Assay.* Adult sea urchins *Paracentrotus lividus* were collected from the Mediterranean Sea at the Cyprus coast and kept in an aerated seawater tank. Gametes were obtained by intracoelomic injection of 0.5 M KCl. Eggs were washed with filtered seawater and fertilized by adding drops of a diluted sperm. Embryos were cultured at room temperature under gentle agitation with a motor-driven plastic paddle (60 rpm) in filtered seawater. The embryos were observed with a light microscope Biolam (LOMO, S.-Petersburg, Russia). For treatment with the test compounds, 5 mL aliquots of embryo suspension were transferred to 6-well plates and incubated as a monolayer at a concentration up to 2000 embryos/mL. Stock solutions of compounds were prepared in DMSO at 5−10 mM concentrations, followed by a 10-fold dilution with 95% EtOH. This procedure enhanced solubility of the test compounds in the salt-containing medium (seawater), as evidenced by microscopic examination of the samples. The maximal tolerated concentrations of DMSO and EtOH in the in vivo assay were determined to be 0.05% and 1%, respectively. Higher concentrations of either DMSO (≥0.1%) or EtOH (>1%) caused nonspecific alteration and retardation of the sea urchin embryo development independent of the treatment stage. Podophyllotoxin (Aldrich) served as reference compound.

The antiproliferative activity was assessed by exposing fertilized eggs (8−20 min after fertilization, 43−55 min before the first mitotic cycle completion) to 2-fold decreasing concentrations of the compound. Cleavage alteration and arrest were clearly detected at 2.5−5.5 h after fertilization. The effects were quantitatively estimated as a threshold concentration resulting in cleavage alteration and embryo death before hatching or full mitotic arrest. At these concentrations, all tested microtubule destabilizers caused 100% cleavage alteration and embryo death before hatching, whereas at 2-fold lower concentrations, the compounds failed to produce any effect. For microtubule destabilizing activity, the compounds were tested on free-swimming blastulae just after hatching (9−10 h after fertilization), originated from the same embryo culture. Embryo spinning was observed after 15 min to 20 h of treatment, depending on the structure and concentration of the compound. Both spinning and lack of forward movement were interpreted to be the result of the tubulin destabilizing activity of a molecule according to previous studies.[20] Video illustrations are available at http://www.chemblock.com. Both sea urchin embryo assay and DTP NCI60 cell line activity data are available free of charge via the Internet at http://www.zelinsky.ru.

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■ ASSOCIATED CONTENT

Supporting Information

Details of experimental procedures and compound characterization data for synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PT, podophyllotoxin; SAR, structure−activity relationship

■ REFERENCES

(1) (a) Hamel, E. Antimitotic natural products and their interactions with tubulin. *Med. Res. Rev.* **1996**, *16*, 207−231. (b) Jordan, A.; Hadfield, J. A.; Laurence, N. J.; McGown, A. T. Tubulin as a target for anticancer drugs: Agents which interact with the mitotic spindle. *Med. Res. Rev.* **1998**, *18*, 259−296. (c) Kingston, D. G. I. Tubulin-interactive natural products as anticancer agents. *J. Nat. Prod.* **2009**, *72*, 507−515.

(2) Kasibhatla, S.; Gourdeau, H.; Meerovitch, K.; Drewe, J.; Reddy, S.; Qiu, L.; Zhang, H.; Bergeron, F.; Bouffard, D.; Yang, Q.; Herich, J.; Lamothe, S.; Cai, S. X.; Tseng, B. Discovery and mechanism of action of a novel series of apoptosis inducers with potential vascular targeting activity. *Mol. Cancer Ther.* **2004**, *3*, 1365−1373.

(3) Kemnitzer, W.; Drewe, J.; Jiang, S.; Zhang, H.; Wang, Y.; Zhao, J.; Jia, S.; Herich, J.; Labreque, D.; Storer, R.; Meerovitch, K.; Bouffard, D.; Rej, R.; Denis, R.; Blais, C.; Lamothe, S.; Attardo, G.; Gourdeau, H.; Tseng, B.; Kasibhatla, S.; Cai, S. X. Discovery of 4-aryl-4*H*-chromenes as a new series of apoptosis inducers using a cell- and caspase-based high-throughput screening assay. 1. Structure-activity relationships of the 4-aryl group. *J. Med. Chem.* **2004**, *47*, 6299−6310.

(4) Kemnitzer, W.; Kasibhatla, S.; Jiang, S.; Zhang, H.; Zhao, J.; Jia, S.; Xu, L.; Crogan-Grundy, C.; Denis, R.; Barriault, N.; Vaillancourt, L.; Charron, S.; Dodd, J.; Attardo, G.; Labrecque, D.; Lamothe, S.; Gourdeau, H.; Tseng, B.; Drewe, J.; Cai, S. X. Discovery of 4-aryl-4*H*-chromenes as a new series of apoptosis inducers using a cell- and caspase-based high-throughput screening assay. 2. Structure−activity relationships of the 7- and 5-, 6-, 8-positions. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4745−4751.

(5) Kemnitzer, W.; Drewe, J.; Jiang, S.; Zhang, H.; Zhao, J.; Crogan-Grundy, C.; Xu, L.; Lamothe, S.; Gourdeau, H.; Denis, R.; Tseng, B.; Kasibhatla, S.; Cai, S. X. Discovery of 4-aryl-4*H*-chromenes as a new series of apoptosis inducers using a cell- and caspase-based high-throughput screening assay. 3. Structure−activity relationships of fused rings at the 7,8-positions. *J. Med. Chem.* **2007**, *50*, 2858−2864.

(6) Kemnitzer, W.; Drewe, J.; Jiang, S.; Zhang, H.; Crogan-Grundy, C.; Labreque, D.; Bubenick, M.; Attardo, G.; Denis, R.; Lamothe, S.; Gourdeau, H.; Tseng, B.; Kasibhatla, S.; Cai, S. X. Discovery of 4-aryl-4*H*-chromenes as a new series of apoptosis inducers using a cell- and caspase-based high throughput screening assay. 4. Structure−activity relationships of N-alkyl substituted pyrrole fused at the 7,8-positions. *J. Med. Chem.* **2008**, *51*, 417−423.

(7) Kemnitzer, W.; Jiang, S.; Wang, Y.; Kasibhatla, S.; Crogan-Grundy, C.; Bubenik, M.; Labrecque, D.; Denis, R.; Lamothe, S.; Attardo, G.; Gourdeau, H.; Tseng, B.; Drewe, J.; Cai, S. X. Discovery of 4-aryl-4*H*-chromenes as a new series of apoptosis inducers using a cell- and caspase-based HTS assay. Part 5: Modifications of the 2- and 3-positions. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 603−607.

(8) Gourdeau, H.; Leblond, L.; Hamelin, B.; Desputeau, C.; Dong, K.; Kianicka, I.; Custeau, D.; Boudreau, C.; Geerts, L.; Cai, S.-X.; Drewe, J.; Labrecque, D.; Kasibhatla, S.; Tseng, B. Antivascular and antitumor evaluation of 2-amino-4-(3-bromo-4,5-dimethoxy-phenyl)-3-cyano-4*H*-chromenes, a novel series of anticancer agents. *Mol. Cancer Ther.* **2004**, *3*, 1375−1384.

(9) Litvinov, Yu. M.; Shestopalov, A. M. Synthesis, structure, chemical reactivity, and practical significance of 2-amino-4*H*-pyrans. In *Advances in Heterocyclic Chemistry*; Katritzky, A. R., Ed.; Elsevier: London, UK, 2011; Vol *103*, Chapter 3, pp 175−260.

(10) (a) Cai, S. X.; Drewe, J.; Kemnitzer, W. Discovery of 4-aryl-4*H*-chromenes as potent apoptosis inducers using a cell- and caspase-based anti-cancer screening apoptosis program (ASAP): SAR studies and the identification of novel vascular disrupting agents. *Anti-Cancer Agents Med. Chem.* **2009**, *9*, 437−456. (b) http://www.epicept.com/Products_/Product_Pipeline/Cancer/EPC2407/ @2012 EpiCept Corporation. All Rights Reserved. (c) ClinicalTrials.gov Identifier: NCT01240590.

(11) (a) Ravelli, R. B. G.; Gigant, B.; Curmi, P. A.; Jourdain, I.; Lachkar, S.; Sobel, A.; Knossow, M. Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain. *Nature* **2004**, *428*, 198−202. (b) Gupta, S.; Das, L.; Datta, A. B.; Poddar, A.; Janik, M. E.; Bhattacharyya, B. Oxalone and lactone moieties of podophyllotoxin exhibit properties of both the B and C rings of colchicine in its binding with tubulin. *Biochemistry* **2006**, *45*, 6467−6475.

(12) Meresse, P.; Dechaux, E.; Monneret, C.; Bertounesque, E. Etoposide: Discovery and medicinal chemistry. *Curr. Med. Chem.* **2004**, *11*, 2443−2466.

(13) Desbene, S.; Giorgi-Renault, S. Drugs that inhibit tubulin polymerization: The particular case of podophyllotoxin and analogues. *Anti-Cancer Agents* **2002**, *2*, 71−90.

(14) Chernysheva, N. B.; Tsyganov, D. V.; Philchenkov, A. A.; Zavelevich, M. P.; Kiselyov, A. S.; Semenov, R. V.; Semenova, M. N.; Semenov, V. V. Synthesis and comparative evaluation of 4-oxa- and 4-aza-podophyllotoxins as antiproliferative microtubule destabilizing agents. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 2590−2593.

(15) Frackenpohl, J.; Adelt, I.; Antonicek, H.; Arnold, C.; Behrmann, P.; Blaha, N.; Böhmer, J.; Gutbrod, O.; Hanke, R.; Hohmann, S.; van Houtdreve, M.; Losel, P.; Malsam, O.; Melchers, M.; Neufert, V.; Peschel, E.; Reckmann, U.; Schenke, T.; Thiesen, H.-P.; Velten, R.; Vogelsang, K.; Weiss, H.-C. Insecticidal heterolignans−tubuline polymerization inhibitors with activity against chewing pests. *Bioorg. Med. Chem.* **2009**, *17*, 4160−4184.

(16) (a) Petcher, T. J.; Weber, H. P.; Kuhn, M.; Von Wartburg, A. Crystal structure and absolute configuration of 2′-bromopodophyllotoxin-0.5 ethyl acetate. *J. Chem. Soc. Perkin Trans. 2* **1973**, *3*, 288−292. (b) Rithner, C. D.; Bushweller, C. H.; Gender, W. J.; Hoogasian, S. Dynamic nuclear magnetic resonance and empirical force field studies of podophyllotoxin. *J. Org. Chem.* **1983**, *48*, 1491−1495.

(17) Shestopalov, A. M.; Shestopalov, A. A.; Rodinovskaya, L. A. Multicomponent reactions of carbonyl compounds and derivatives of cyanoacetic acid: synthesis of carbo- and heterocycles. *Synthesis* **2008**, *1*, 1−25.

(18) Raghuvanshi, D. S.; Singh, K. N. An expeditious synthesis of novel pyranopyridine derivatives involving chromenes under controlled microwave irradiation. *ARKIVOC* **2010**, No. x, 305−317.

(19) Tietze, L. F.; Brasche, G.; Gericke, K. M. *Domino Reactions in Organic Synthesis*; Wiley-VCH: Weinheim, Germany, 2006; 617 p.

(20) Semenova, M. N.; Kiselyov, A. S.; Semenov, V. V. Sea urchin embryo as a model organism for the rapid functional screening of tubulin modulators. *BioTechniques* **2006**, *40*, 765−774.
(21) (a) Semenova, M. N.; Kiselyov, A. S.; Titov, I. Y.; Raihstat, M. M.; Molodtsov, M.; Grishchuk, E.; Spiridonov, I.; Semenov, V. V. In Vivo evaluation of indolyl glyoxamides in the sea urchin embryo model: correlation with *in vitro* tubulin dynamics effects. *Chem. Biol. Drug Des.* **2007**, *70*, 485−490. (b) Kiselyov, A. S.; Semenova, M. N.; Chernyshova, N. B.; Leitao, A.; Samet, A. V.; Kislyi, K. A.; Raihstat, M. M.; Oprea, T.; Lemcke, T.; Lantowe, M.; Weiss, D. G.; Ikizalp, N. N.; Kuznetsov, S. A.; Semenov, V. V. Novel derivatives of 1,3,4-oxadiazoles are potent mitostatic agents featuring strong microtubule depolymerizing activity in the sea urchin embryo and cell culture assays. *Eur. J. Med. Chem.* **2010**, *45*, 1683−1697.
(22) Magedov, I. V.; Frolova, L.; Manpadi, M.; Bhoga, U. D.; Tang, H.; Evdokimov, N. M.; George, O.; Georgiou, K. H.; Renner, S.; Getlik, M.; Kinnibrugh, T. L.; Fernandes, M. A.; van Slambrouck, S.; Steelant, W. F. A.; Shuster, C. B.; Rogelj, S.; van Otterlo, W. A. L.; Kornienko, A. Anticancer properties of an important drug lead podophyllotoxin can be efficiently mimicked by diverse heterocyclic scaffolds accessible via one-step synthesis. *J. Med. Chem.* **2011**, *54*, 4234−4246.
(23) Semenova, M. N.; Kiselyov, A. S.; Tsyganov, D. V.; Konyushkin, L. D.; Firgang, S. I.; Semenov, R. V.; Malyshev, O. R.; Raihstat, M. M.; Fuchs, F.; Stielow, A.; Lantow, M.; Philchenkov, A. A.; Zavelevich, M. P.; Zefirov, N. S.; Kuznetsov, S. A.; Semenov, V. V. Polyalkoxybenzenes from plants. 4. Dill and parsley seed extracts in synthesis of azapodophyllotoxins featuring strong tubulin destabilizing activity in the sea urchin embryo and cell culture assays. *J. Med. Chem.* **2011**, *54*, 7138−7149.
(24) Cai, S. X.; Drewe, J. A.; Kasibhatla, S.; Kemnitzer, W. D.; Tseng, B. Y.; Blais, C.; Labreque, D.; Gourdeau, H. Substituted 4*H*-chromenes and analogs as activators of caspases and inducers of apoptosis and the use thereof. U.S. Patent 7,968,595 B2, 2011.