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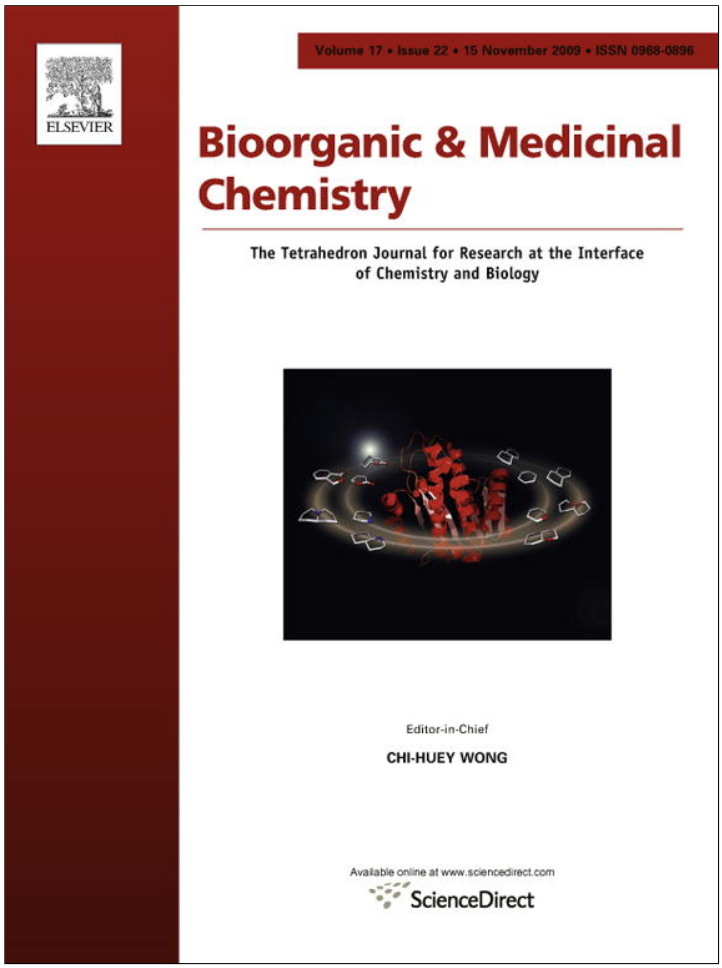

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Combretastatin-like chalcones as inhibitors of microtubule polymerization. Part 1: Synthesis and biological evaluation of antivascular activity

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ABSTRACT

The α-methyl chalcone SD400 is a potent inhibitor of tubulin assembly and possesses potent anticancer activity. Various chalcone analogues were synthesized and evaluated for their cell growth inhibitory properties against the K562 human chronic myelogenous leukemia cell line (SD400, IC_{50} 0.21 nM; combretastatin A4 CA4, IC_{50} 2.0 nM). Cell cycle analysis by flow cytometry indicated that these agents are antimitotic (SD400, 83% of the cells are in G_2/M phase; CA4 90%). They inhibit tubulin assembly at low concentration (SD400, IC_{50} 0.46 μM; CA4, 0.10 μM) and compete with [^{3}H]colchicine for binding to tubulin (8% [^{3}H]colchicine remained bound to tubulin after competition with SD400 or CA4). Upon treatment with SD400, remarkable cell shape changes were elicited in HUVEC cells, consistent with vasculature damaging activity.

1. Introduction

The combretastatins are a group of antimitotic agents isolated from the bark of the South African tree *Combretum caffrum*.[1] The most active of these, combretastatin A4 (**1**) (CA4), is a potent cytotoxic agent that strongly inhibits the polymerization of tubulin by binding to the colchicine-binding site of the β-tubulin subunit.[2] Combretastatin A4 has provided a simple structural template for the design of related compounds with potent biological activity and a large number of combretastatin-type analogues have been prepared and assessed as potential anticancer agents, some of which have recently been reviewed.[3–6] Further interest in combretastatin derivatives was stimulated by the discovery that CA4 is also able to elicit irreversible vascular shutdown within solid tumors, leaving normal vasculature intact.[7,8] In this way tumors are starved of oxygen and nutrients and their constituent cells die. Agents that act in this manner have the potential to make a significant impact on the clinical management of cancer.[9] Tubulin therefore remains an important target for the design of anticancer agents. The antivascular effect of these agents derives from the role that tubulin and microtubules play in determining the elongated shape of vascular endothelial cells. The cellular microtubule network, a principal part of the cytoskeleton, plays a major role in maintaining cell shape, particularly in the case of neovasculature. It has been shown that endothelial cells of immature vasculature have a less developed actin cytoskeleton and are therefore more sensitive to the effects of anti microtubular agents.[10] Agents such as CA4 cause microtubule disassembly and consequently the endothelial cells of immature vasculature collapse and very quickly block blood flow through the tumor vascular network. This effect is most pronounced for agents that interact with the colchicine-binding site. It must be emphasized that the therapeutic effects of these agents such as CA4 are derived from their vasculature targeting properties and not antimitotic properties. A prodrug of combretastatin A4, the water-soluble phosphate derivative CAP (Zybrestat™) is now in phase III clinical trials for the treatment of cancer.[11]

Our own program of research of CA4 and surrogates[12–16] has included the chalcones, which can be viewed simplistically as keto-stilbenes.[17–19] Chalcones are known to possess a range of interesting biological activity[20,21] and some have been investigated for their anticancer properties.[22] Attention to the antimitotic

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properties was first drawn by Edwards et al.[23] As part of our studies, we have incorporated the aryl substitution pattern of CA4 into chalcones and have shown that **2a** and **3a** possess potent antimitotic properties.[18,19,24] We now report full details of the design, synthesis, and biological assessment of these and further chalcone derivatives.

2. Results and discussion

2.1. Chemistry

The chalcones **2** (1,3-diarylprop-2-en-1-ones) were prepared in good yields using the Claisen–Schmidt condensation (Scheme 1, Table 1). This method for the preparation of chalcones is attractive since it predominantly generates the (*E*)-isomer from simple building blocks. A large number of these substituted benzaldehydes and acetophenones are commercially available and inexpensive.

Chalcones **3** possessing an alkyl group at the α-position (C-2) were prepared by the piperidinium acetate catalyzed Knoevenagel-like condensation of substituted propiophenones and the appropriate substituted benzaldehyde.[23] The substituted propiophenones were prepared by addition of an alkylmagnesium bromide to the appropriate benzaldehyde, followed by Swern or PCC oxidation of the intermediate alcohols. Chalcones **3** (Scheme 1, Table 2) were usually obtained in modest yields as a mixture of geometrical isomers (usually *E*:*Z*, 5:1), which were separated by column chromatography and purified by recrystallization.

Chalcones **4** possessing an alkoxyl group at the α-position were prepared in a similar way by combination of a substituted α-alkoxyacetophenones with appropriate substituted benzaldehydes. The substituted α-alkoxyacetophenones were prepared by photochemical α-bromination of acetophenones, followed by silver-catalyzed alkoxide substitution of the halide of intermediate phenacyl bromide.[25] Higher yields of **4** within this series were observed for the fluorinated benzaldehydes (Scheme 1, Table 3).

The importance of the enone unit was probed by functionalization of the chalcones. A series of epoxides **5** was prepared by base-promoted reaction of chalcones **2** with *t*-butyl hydroperoxide.[26] The epoxides **5** were obtained as racemates in high yields (Scheme 1, Table 4). Several of the chalcones **2 and 3** were reduced with sodium borohydride in the presence of cerium chloride to afford alcohol **6**.[33] Hydrogenation of the chalcones produced a mixture of alcohol **7**, ketone **8**, and diarylpropane **9**. These products proved easy to separate by flash chromatography (Scheme 1, Table 5).

A desirable feature for an anticancer drug is partial solubility in aqueous media. Phosphate prodrugs have already proven to be successful in the case of CA4.[27] Phosphate prodrugs **10** were prepared from chalcones **3a** and **4a** (Scheme 2, Table 6). Di-*tert*-butyl *N*,*N*-diethylphosphoramidite is a highly reactive phosphitylating agent, which, on activation with 1*H*-tetrazole, reacted rapidly with chalcones **3a** and **4a**. Subsequent oxidation of the resultant aryl di-*tert*-butyl phosphites **10** with *m*-CPBA gave the aryl di-*tert*-butyl phosphates **11**. Cleavage of the *t*-butyl groups was achieved by hydrolysis with 5 M hydrochloric acid/dioxane and the resultant 3′-phosphoryl chalcones **12** were converted to the disodium phosphates **13** by passage through a Dowex-50W-X8 (Na^+) cation-exchange column.[28]

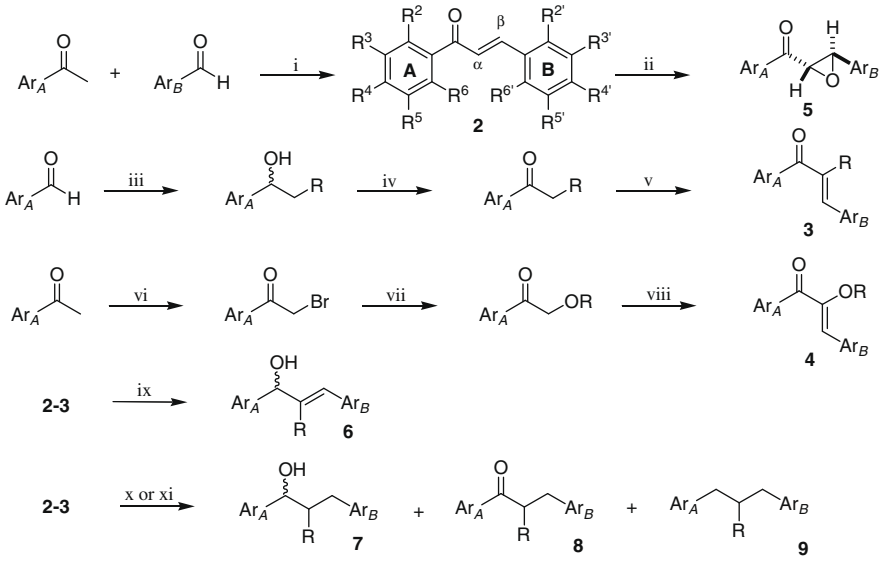
2.2. Biological evaluation

2.2.1. Cancer cell growth inhibitory studies

The growth inhibitory properties of the chalcones were determined in vitro using the MTT assay.[29] The drugs were screened against the K562 human chronic myelogenous leukemia cell line. The results are depicted in Tables 1–6 as an IC_{50} (μM), the concentration required to inhibit 50% cell proliferation.

The most potent chalcones bear the CA4 **1** aromatic substitution pattern and display impressive activities in the nanomolar range (**3a** 0.21, **4a** 1.5, and **2a** 4.3 nM). By comparison the parent unsubstituted chalcone (**2ee**) displayed only modest activity (3.8 μM).

In general, the activities were highly dependant on the aromatic substitution pattern and methoxylated chalcones exhibited highest activities. The most active chalcones were usually obtained when at least one of the rings possessed three methoxyl groups, regardless of their positions. Where only moderate activities were observed when both rings were monosubstituted (**2yy** 15 μM), disubstituted (**2z** 2.2 μM), and trisubstituted (**2f** 0.2 μM, **2t** 1.5 μM) methoxyl groups on both rings greatly increased activity.



Scheme 1. Reagents and conditions: (i) NaOH, MeOH, rt, overnight; (ii) *t*-BuOOH, $PhCH_2NMe_3^+OH^-$, benzene, rt, overnight; (iii) (a) RCH_2MgBr, THF, reflux, 1 h, (b) 1 N HCl; (iv) DMSO, DCM, oxalyl chloride, −78 °C, 1 h then rt, 2 h or PCC, DCM, rt, 3 h; (v) ArBCHO, piperidine, AcOH, dry ethanol, Soxhlet with 4 Å molecular sieves, reflux, 2 days; (vi) Br_2, *hν*, dry Et_2O, 0 °C, 1 h; (vii) ROH, Ag_2CO_3, $BF_3{\cdot}Et_2O$, rt, 2 days; (viii) ArBCHO, NaOH, MeOH, rt, overnight; (ix) $NaBH_4$, $CeCl_3{\cdot}7H_2O$, MeOH, H_2O, 0 °C, 4 h; (x) H_2 (1 atm), 10% Pd/C, EtOAc, rt, 5 h; (xi) H_2 (1 atm), 10% Pd/C, DCM, rt, 30 min.

Table 1
Structures, yields of chalcones **2** from aldol condensation and inhibition of cell proliferation

Compds	R^2	R^3	R^4	R^5	R^6	$R^{2'}$	$R^{3'}$	$R^{4'}$	$R^{5'}$	$R^{6'}$	Yield (%)	IC_{50}[a] (μM)
2a	H	OMe	OMe	OMe	H	H	OH	OMe	H	H	58	0.0043
2b	H	OMe	OMe	OMe	H	H	NH_2	OMe	H	H	32[b]	0.01
2c	OMe	OMe	OMe	H	H	H	OH	OMe	H	H	66	0.01
2d	OMe	H	H	OMe	H	H	H	NMe_2	H	H	78	0.04
2e	H	OMe	OMe	OMe	H	H	O–$(CH_2)_2$–O		H	H	94	0.08
2f	H	OMe	OMe	OMe	H	OMe	H	OMe	H	OMe	72	0.20
2g	H	OMe	OMe	OMe	H	H	OMe	OMe	H	H	90	0.30
2h	H	OMe	OMe	OMe	H	H	O–CH_2–O		H	H	73	0.30
2i	H	OMe	OMe	OMe	H	H	F	OMe	H	H	69	0.30
2j	H	OMe	OMe	OMe	H	H	H	OMe	H	H	91	0.30
2k	H	OMe	OMe	H	H	OMe	H	OMe	H	OMe	80	0.30
2l	OMe	H	OMe	H	OMe	H	OMe	OMe	OMe	H	64	0.30
2m	OMe	OMe	OMe	H	H	H	H	OMe	H	H	86	0.30
2n	OMe	H	H	OMe	H	H	O–$(CH_2)_2$–O		H	H	85	0.30
2o	OMe	OMe	OMe	H	H	H	F	OMe	H	H	74	0.35
2p	H	O–CH_2–O		H	H	OMe	H	OMe	OMe	OMe	58	1.1
2q	H	OMe	OMe	H	H	H	OMe	OMe	OMe	H	84	1.1
2r	H	OMe	OMe	OMe	H	H	OH	OH	H	H	34	1.2
2s	H	H	Me	H	H	H	OH	OMe	H	H	76	1.5
2t	H	OMe	OMe	OMe	H	H	OMe	OMe	OMe	H	62	1.5
2u	H	OMe	OMe	OMe	H	H	H	Cl	H	H	55	1.5
2v	H	OMe	OMe	OMe	H	H	H	F	H	H	70	1.5
2w	H	H	H	H	H	H	OMe	OMe	OMe	H	63	1.6
2x	H	H	F	H	H	H	OMe	OMe	OMe	H	83	1.6
2y	OMe	OMe	OMe	H	H	H	OH	OH	H	H	38	1.7
2z	H	OMe	OMe	H	H	H	OMe	OMe	H	H	88	2.2
2aa	OMe	H	OMe	H	H	H	H	OMe	H	H	93	2.7
2bb	H	O–CH_2–O		H	H	H	OMe	OMe	OMe	H	76	2.9
2cc	H	OMe	OH	H	H	H	OMe	OMe	OMe	H	41	3.5
2dd	H	OMe	OMe	OMe	H	Cl	H	Cl	H	H	83	3.8
2ee	H	H	H	H	H	H	H	H	H	H	–	3.8
2ff	F	H	OMe	H	H	OMe	H	H	OMe	H	68	4.0
2gg	H	OMe	OMe	OMe	H	H	NO_2	OMe	H	H	61	4.0
2hh	OH	H	OMe	H	H	H	OMe	OMe	OMe	H	52	4.4
2ii	H	H	OMe	H	H	H	OMe	OMe	OMe	H	82	4.5
2jj	OMe	OMe	OMe	H	H	H	F	OMe	F	H	79	4.8
2kk	H	H	F	H	H	H	H	F	H	H	84	4.9
2ll	H	H	F	H	H	H	O–CH_2–O		H	H	90	5.1
2mm	H	O–$(CH_2)_2$–O		H	H	H	OMe	OMe	OMe	H	72	5.6
2nn	H	OMe	OMe	OMe	H	H	F	OMe	F	H	72	6.2
2oo	H	O–CH_2–O		H	H	OMe	H	H	OMe	H	63	6.4
2pp	H	O–CH_2–O		H	H	H	OMe	OMe	H	H	77	7.1
2qq	OMe	H	OMe	H	OMe	OMe	H	H	OMe	H	90	7.2
2rr	H	O–$(CH_2)_2$–O		H	H	H	O–CH_2–O		H	H	92	8.0
2ss	H	H	OH	H	H	H	OMe	OMe	OMe	H	93	8.9
2tt	OH	H	OMe	H	H	H	H	Cl	H	H	72	9.7
2uu	H	H	OMe	H	H	H	H	OMe	H	H	66	9.9
2vv	H	OMe	OMe	OMe	H	H	H	OBn	H	H	42	9.9
2ww	H	OMe	OMe	H	H	H	H	OMe	H	H	87	10
2xx	H	H	Me	H	H	H	H	Me	H	H	88	11
2yy	H	H	OMe	H	H	H	H	OMe	H	H	71	15
2zz	OH	H	OMe	H	H	H	OMe	OMe	H	H	45	16
2α	H	H	Me	H	H	H	H	OMe	H	H	97	20
2β	H	H	Me	H	H	H	OMe	OMe	OMe	H	83	24

[a] Drug concentration that inhibits the growth of K562 cell line by 50% after incubation for 5 days. Each drug concentration was tested in triplicate, and the standard error of each value in <10%.
[b] The yield relates to the reduction ($SnCl_2 \cdot 2H_2O$, concd HCl, EtOH:EtOAc, 1:1, reflux, 2 days) of chalcone **2gg** to provide chalcone **2b**.

Replacement of hydroxyl groups by a fluorine atom was tolerated (OH: **2a** 4.3 nM, **3a** 0.21 nM, **4a** 1.5 nM vs F: **2i** 0.3 μM, **3c** 2.0 nM, **4c** 3.7 nM). The latter two chalcones serve as prototypes that will not undergo metabolism via conjugation of the phenolic OH group.[13]

Striking improvement in potency (2–20-fold) was observed when the chalcones were substituted in the alpha position with alkyl (Me: **3a** 0.21 nM, Pr: **3b** 2.2 nM) or alkoxyl groups (OCH_3: **4a** 1.5 nM, OEt: **4b** 2.6 nM) instead of the hydrogen (H: **2a** 4.3 nM).[24]

The chalcone epoxides displayed modest activity, as did most of the reduced analogues. It is interesting that the saturated ketone **8a** (30 nM) and allylic alcohol **6a** (90 nM) are significantly cytotoxic. We believe that the absence of the conjugated α,β-unsaturated linkage, introduces conformational flexibility that is unfavorable for activity. It is unlikely that the chalcones are acting as Michael acceptors since **8a** remains considerably active. The phosphate prodrugs **13b** (0.12 nM) and **13a** (8.0 nM) were found to be equipotent to their 'parent' drugs **3a** and **2a**, respectively, which is consistent with their design.

2.2.2. Cell cycle analysis

Flow cytometry provides a practical method for the analysis of a wide range of cellular components. In this study, the technique was used to evaluate the effects of agents on the growth and

Table 2
Structures, yields of α-R chalcones **3** and inhibition of cell proliferation

Compds	R	R^2	R^3	R^4	R^5	R^6	$R^{2'}$	$R^{3'}$	$R^{4'}$	$R^{5'}$	$R^{6'}$	Yield (%)	IC_{50}[a] (μM)
3a	Me	H	OMe	OMe	OMe	H	H	OH	OMe	H	H	50	0.00021
3b	Pr	H	OMe	OMe	OMe	H	H	OH	OMe	H	H	44	0.0022
3c	Me	H	OMe	OMe	OMe	H	H	F	OMe	H	H	19	0.0020
3d	Me	H	OMe	OMe	OMe	H	H	$O–(CH_2)_2–O$	H	H	35	0.011	
3e	Me	OMe	H	H	OMe	H	H	H	NMe_2	H	H	20	0.012
3f	Me	H	OMe	OMe	OMe	H	H	$O–CH_2–O$	H	H	35	0.014	
3g	Me	H	OMe	OMe	OMe	H	H	F	OMe	F	H	62	0.050
3h	Me	H	OMe	OMe	OMe	H	H	NO_2	OMe	H	H	51	0.060
3i	Me	H	H	OMe	H	H	H	H	OMe	H	H	72	1.9
3j	Me	H	H	OMe	H	H	OMe	H	H	OMe	H	66	2.3
3k	Me	H	OMe	OMe	OMe	H	H	OMe	OMe	OMe	H	34	2.5
3l	Me	H	OMe	OMe	OMe	H	OMe	H	OMe	H	OMe	28	>25

[a] Drug concentration that inhibits the growth of K562 cell line by 50% after incubation for 5 days. Each drug concentration was tested in triplicate, and the standard error of each value in <10%.

Table 3
Structures, yields of α-OR chalcones **4** and inhibition of cell proliferation

Compds	R	R^2	R^3	R^4	R^5	R^6	$R^{2'}$	$R^{3'}$	$R^{4'}$	$R^{5'}$	$R^{6'}$	Yield (%)	IC_{50}[a] (μM)
4a	Me	H	OMe	OMe	OMe	H	H	OH	OMe	H	H	31	0.0015
4b	Et	H	OMe	OMe	OMe	H	H	OH	OMe	H	H	32	0.0026
4c	Me	H	OMe	OMe	OMe	H	H	F	OMe	H	H	62	0.0037
4d	Et	H	OMe	OMe	OMe	H	H	F	OMe	H	H	54	0.011
4e	Pr	H	OMe	OMe	OMe	H	H	F	OMe	H	H	61	0.020
4f	Pr	H	OMe	OMe	OMe	H	H	F	OMe	F	H	70	0.22
4g	Et	H	OMe	OMe	OMe	H	H	F	OMe	F	H	69	0.23
4h	Me	H	OMe	OMe	OMe	H	H	F	OMe	F	H	75	0.36

[a] Drug concentration that inhibits the growth of K562 cell line by 50% after incubation for 5 days. Each drug concentration was tested in triplicate, and the standard error of each value in <10%.

Table 4
Structures, yields of epoxide chalcones **5** and inhibition of cell proliferation

Compd	R^2	R^3	R^4	R^5	R^6	$R^{2'}$	$R^{3'}$	$R^{4'}$	$R^{5'}$	$R^{6'}$	Yield (%)	IC_{50}[a] (μM)
5a	H	OMe	OMe	OMe	H	H	H	Me	H	H	83	3.4
5b	H	OMe	OMe	OMe	H	H	OMe	OMe	OMe	H	69	3.7
5c	H	OMe	OMe	OMe	H	H	H	OMe	H	H	84	4.9
5d	H	H	Me	H	H	H	H	Me	H	H	62	12
5e	H	H	Me	H	H	H	H	OMe	H	H	51	15
5f	H	H	OMe	H	H	H	OMe	OMe	H	H	71	>30
5g	H	H	OMe	H	H	H	H	OMe	H	H	82	>30
5h	H	H	OMe	H	H	H	H	Me	H	H	83	>30

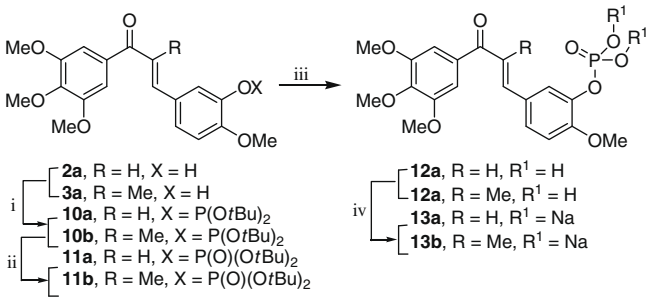
[a] Drug concentration that inhibits the growth of K562 cell line by 50% after incubation for 5 days. Each drug concentration was tested in triplicate, and the standard error of each value in <10%.

Table 5
Structures, yields of reduced chalcones **6**–**9** and inhibition of cell proliferation

Compds	R	R^2	R^3	R^4	R^5	R^6	$R^{2'}$	$R^{3'}$	$R^{4'}$	$R^{5'}$	$R^{6'}$	Yield (%)	IC_{50}[a] (μM)
8a	Me	H	OMe	OMe	OMe	H	H	OH	OMe	H	H	57[b]	0.03
6a	Me	H	OMe	OMe	OMe	H	H	OH	OMe	H	H	99[c]	0.09
6b	H	H	OMe	OMe	OMe	H	H	OH	OMe	H	H	54[c]	0.5
9a	H	H	OMe	OMe	OMe	H	H	H	OMe	H	H	33[b]	0.5
7a	Me	H	OMe	OMe	OMe	H	H	OH	OMe	H	H	44[b]	0.6
7b	H	H	OMe	OMe	OMe	H	H	OH	OMe	H	H	15[b]	1.0
8b	H	H	OMe	OMe	OMe	H	H	OH	OMe	H	H	68[b]	1.0
7c	H	H	OMe	OMe	OMe	H	H	H	OMe	H	H	18[b]	4.0
6c	H	H	OMe	OMe	OMe	H	H	H	OMe	H	H	99[c]	4.0
8c	H	H	OMe	OMe	OMe	H	H	H	OMe	H	H	81[b]	10
8d	H	H	H	OMe	H	H	H	OMe	OMe	OMe	H	50[b]	12

[a] Drug concentration that inhibits the growth of K562 cell line by 50% after incubation for 5 days. Each drug concentration was tested in triplicate, and the standard error of each value in <10%.
[b] Using conditions IX in Scheme 1.
[c] Using conditions X or XI in Scheme 1.

division of cells, by measuring the DNA content of eukaryotic cells. The results are plotted as a histogram, showing the proportion of cells emitting a given level of fluorescent proportional to the DNA content. This method gives a rapid and accurate measure of



Scheme 2.

Table 6
Structures, yields of chalcone prodrugs **10**–**11** and inhibition of cell proliferation

Compds	IC_{50}[a] (μM)
13b	0.00012
13a	0.0080
12b	0.5
12a	0.3

[a] Drug concentration that inhibits the growth of K562 cell line by 50% after incubation for 5 days. Each drug concentration was tested in triplicate, and the standard error of each value in <10%.

the cell cycle effects of anticancer agents. A typical histogram for antimitotic agent **3a** is shown in Figure 1. The results are depicted numerically in Table 7.

It is evident that the cell population of the untreated cells predominantly resides in the G_0–G_1/S-phases of the cycle (93%). In stark contrast the cells treated with selected chalcones block the cells in mitosis as illustrated through an accumulation of cells in the G_2/M phase (19–90% compared to 7% for untreated cells). Such cells appear to have the capacity to replicate their DNA but are not able to proceed through the cell cycle to cell division. These results are consistent with those obtained for classical tubulin-binding drugs.[30] We note that chalcones that exhibited the greatest cell growth inhibitory properties block the cell cycle in the G_2/M phase most effectively (**3c** 88%, **4a** 84%, **3e** 83%, **2a** 82%).

2.2.3. Immunohistochemistry assay

The strong cell growth inhibitory properties of selected chalcones supported by their significant G_2/M blocking properties prompted us to focus on tubulin as a potential molecular target. Immunohistochemistry enables the visualization of intracellular distribution of microtubules following treatment with a drug through the application of a suitable set of antibodies.

The intracellular network of microtubules is seen clearly in the untreated cells (Fig. 2a), irradiating from the center towards the perimeter of the cell. Such an organized system is destroyed (Fig. 2b) following treatment with selected agents **2e**, **2f**, **2k**, **3a**, **3c**, **3e**, and **4a**. These agents result in the observation of a diffuse staining pattern and a granular cytoplasm in comparison to the

Table 7
Flow cytometry (cell cycle distribution, % cells in G_0–G_1, S, G_2/M phases), inhibition of tubulin assembly (TA), and binding of [^{3}H]colchicine to tubulin (% [^{3}H]colchicine remaining bound to tubulin after competition)

Compd	Flow cytometry			TA IC_{50}[a] (μM)	% [^{3}H]Col bound[b]
	G_0–G_1	S	G_2/M		
Control	48	45	7	N/A	N/A
1	2	8	90	0.1	8
3c	3	9	88	0.65	6
4a	4	12	84	0.51	5
3a	10	7	83	0.46	8
3e	10	8	82	10	2
2a	12	12	76	0.62	9
2l	10	16	74	15	27
2f	10	18	72	12	46
2i	9	19	72	1.2	N.D.[c]
3g	4	24	72	2.4	25
13b	21	10	69	3.1	12
13a	22	14	64	39	83
2nn	20	19	61	12	N.D.[c]
4h	30	15	55	1.7	41
2e	27	23	50	13	10
2o	37	14	49	7.9	N.D.[c]
2k	26	27	47	18	42
2jj	51	3	46	31	N.D.[c]
4c	41	13	46	0.47	8
2g	31	25	44	>25	62
2c	32	28	40	0.45	N.D.[c]
8a	36	36	28	4.0	N.D.[c]
3h	36	36	28	>10	N.D.[c]
6a	44	33	24	>10	N.D.[c]
2b	48	33	19	2.0	N.D.[c]

[a] Tubulin concentration was 1 mg/mL, IC_{50} concentration of drug to inhibit 50% of tubulin assembly.
[b] Individual assays were performed in triplicate samples.
[c] Not determined.

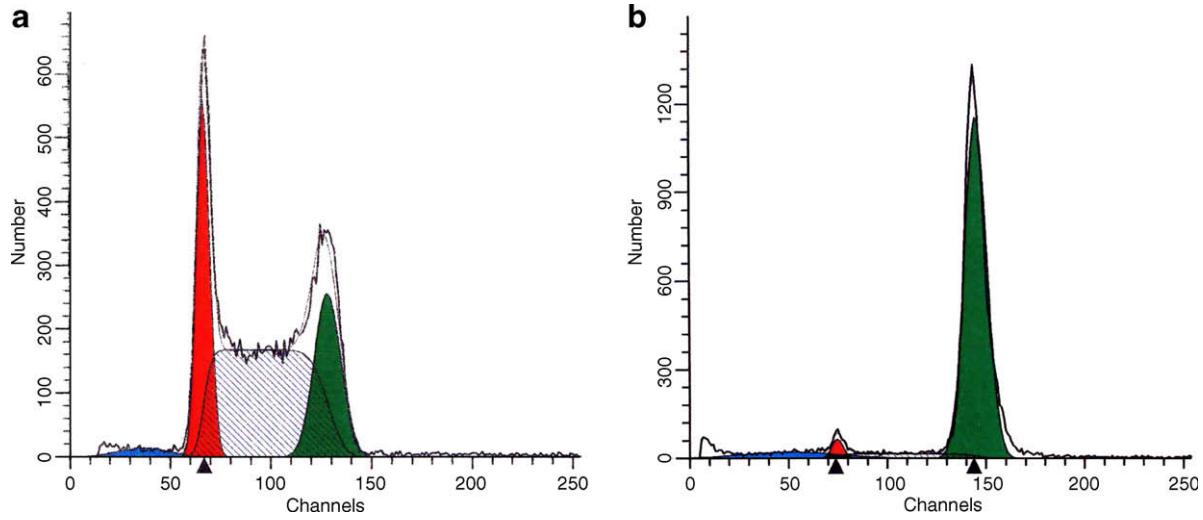


Figure 1. Cell cycle distribution by flow cytometry in K562 cells treated for 24 h with (a) control (b) 20 nM chalcone **3a**.

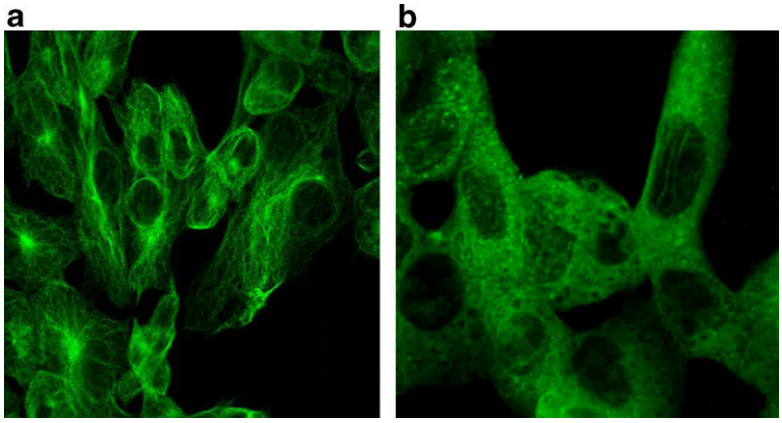


Figure 2. Induction of morphological change in VERO cells. VERO cells were incubated without (a) and with (b) **4a**.

control cells. Such morphological changes are indicative of depolymerized, dispersed tubulin dimers and are very similar to the reported cellular changes caused by treatment with colchicine.[31]

2.2.4. Tubulin assembly assay

The latter biological results prompted us to study the effects upon in vitro assembly of tubulin of selected chalcones. Purified tubulin assembles at temperature above 30 °C in the presence of GTP and magnesium ions. The assembly is accompanied by an increase in turbidity which can be measured spectrophotometrically at λ 350 nm. Drugs that inhibit tubulin assembly produce a lower change in optical density upon warming, compared to an untreated control. The results are shown in Table 7.

In general, cell growth inhibitory and G_2/M blocking properties correlated with the inhibition of tubulin assembly. As noted previously by Cushman et al.,[32] the cell growth and tubulin assembly inhibitory properties are not in the same range. It is clear that chalcones resembling CA4 offer the highest anti-tubulin assembly activity, in the sub-micromolar range (<1 μM). The 3,4,5-trimethoxyl A-ring arrangement and hydroxyl group at the 3-position on ring B, as found in compounds **2a**, **3a**, and **4a**, are clearly advantageous in the ability of a drug to inhibit tubulin assembly and illustrate their overriding importance in binding to tubulin. As anticipated the phosphate prodrugs were found to be inactive as tubulin assembly inhibitors. As this is not a cell-based assay, the prodrugs are not subjected to in vitro hydrolysis by phosphatases.

2.2.5. [^{3}H]colchicine competition assay

To support the former results, we examined the ability of selected chalcones to compete with colchicine for binding to tubulin. Radiolabeled [^{3}H]colchicine and the drugs were allowed to competitively bind to tubulin. The samples were passed through a column and the eluent containing unbound [^{3}H]colchicine was analyzed by scintillation spectroscopy. The results are depicted in Table 7.

Again, as observed in previous assays, the CA4-like chalcones **2a**, **3a**, and **4a** strongly displaced colchicine from its binding site on tubulin (<10% [^{3}H]colchicine remained bound to tubulin). The most active chalcone **3e** left only 2% colchicine bound to tubulin. As anticipated, the phosphate prodrugs were inactive in the colchicine competition assay. Since this is not a cell-based assay, the drug is not subject to cellular phosphatases. Therefore, the bulky substituent at the 3-position of the B ring leads to low affinity for the colchicine-binding site of tubulin.

2.2.6. Electron microscopy

Having shown that the selected chalcones inhibit tubulin assembly and strong competitors of colchicine we used electron microscopy to study further their effects upon the structure of polymerized tubulin. Tubulin was assembled on a carbon-coated copper grid in the presence of GTP and chalcone **2a** and **3a** at 37 °C. The grids were then rinsed with distilled water and negatively stained with uranyl acetate before they were viewed in the scanning electron microscope and photographed.

Negative staining of isolated untreated tubulin demonstrated parallel, longitudinal profilaments, made up of subunits with a diameter close to 5 nm (Fig. 3a). The flattened microtubules (MT) showed six to seven profilaments, with evidence of beading, shown by a partial elliptical opening of the MT, clearly suggesting a regular tubular MT made up of 13 profilaments.

MT structures were found to be altered by chalcone **2a** showing 100 nm long MTs, which appeared a lot shorter than the untreated MTs (Fig. 3b). The MTs appear twisted, which made the counting of the number of subunit impossible. When treated with **3a**, the negatively stained pictures showed short MTs as well as rings (Fig. 3c). Enlargement of the picture allowed us to measure the inner diameter (25 nm vs 15 nm for normal MT) and outer diameter (50 nm vs 25 nm for normal MT), suggesting the formation of a double ring and a bigger cross-section containing possibly 15 subunits in the inner ring and 21 subunits in the outer ring. Studies carried out on the ring formation in relation with MT assembly suggested several possibilities. Some suggested that the rings, associated with microtubule associated protein MAP2, would fragment into oligomers from which MT would be assembled.[33] Others believe that rings are not assembly intermediates for tubulin, but that an equilibrium between dimers and rings would exist and that the ring may result directly from MT disassembly.[34] More recently Nogales and co-workers suggested that agents that bound to the colchicine-binding site did so at the α,β-interface which tilted the dimer by 10°, eventually allowing a protofilament to close into a ring structure.[35,36] Whatever the reason, the effects upon microtubule morphology are dramatic and clearly different for the two agents.

2.2.7. HUVEC permeability assay

CA4 **1** has been shown to induce vascular-mediated tumor necrosis in animal models.[8] Since our chalcones seem to act in a similar manner to CA4, their antivascular properties were evaluated using the HUVEC (Human Umbilical Vascular Epithelial Cell) permeability assay.[37] This assay measures, in a semi-quantitative manner, alteration in cell shape. The HUVECs were grown on a porous filter within a hollow chamber, forming a confluent layer across the permeable surface. The assay measures the passage of a dextran marker across the barrier of the cells. Cells that have been the subject of cellular shape change upon incubation with a candidate drug, will provide 'channels' for the dextran marker to pass through. A blank chamber, void of cells, allows maximum

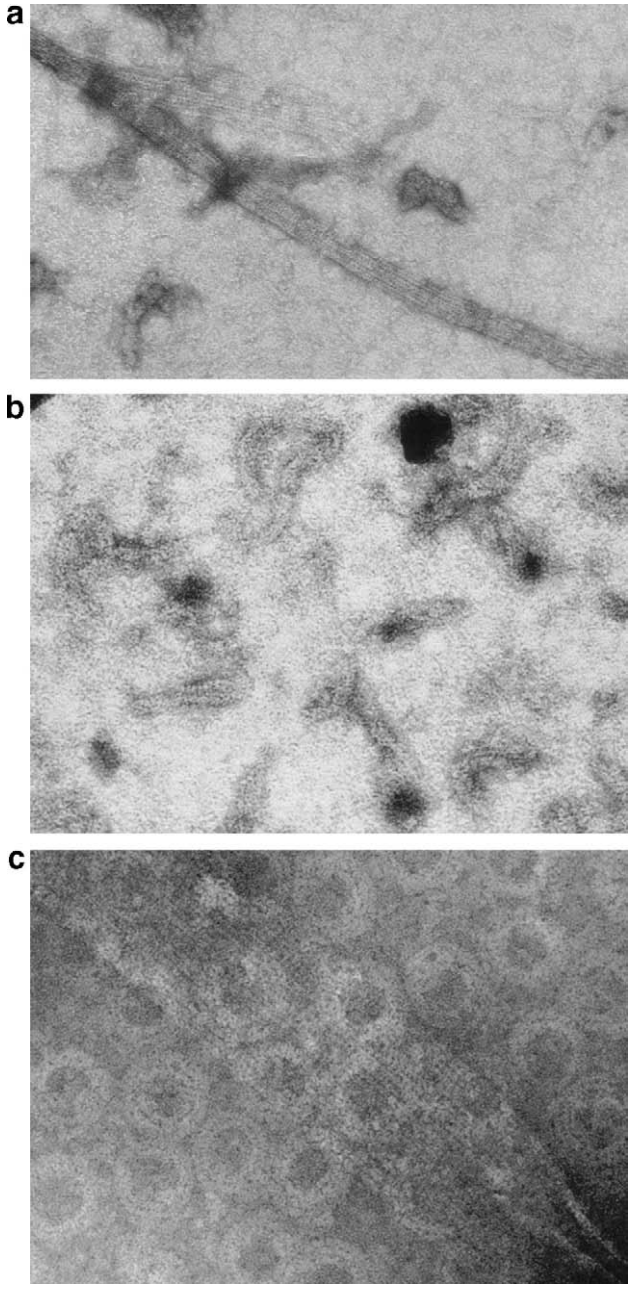


Figure 3. Scanning electron micrographs of negatively stained microtubules (a) untreated, (b) treated with **2a**, (c) treated with **3a**.

passage of the marker through the disk and a surface bearing a confluent layer of cells exclusively inhibits any movement. We employ combretastatin A4 **1** as a positive control and methotrexate (MTX) as a negative control in the assay.

It is evident from the data (Fig. 4) that drugs **2a** and **3a**, along with their water-soluble prodrugs **13a** and **13b**, respectively, elicited remarkable cell shape change, illustrated through the detection of large quantities of the marker having passed through the barrier of cells. The magnitude of shape change is significant. The values obtained for **2a**/**3a** and **13a**/**13b** appear better than those of CA4 **1** and its disodium phosphate salt **1a**, which have in vivo vascular damaging properties. These properties are derived, in part, from their ability to elicit endothelial cell shape change. The α-methoxy-chalcones **4a**, **4c,** and **4h** display slightly lower levels of activity, but nevertheless they are equipotent to CA4 **1**.

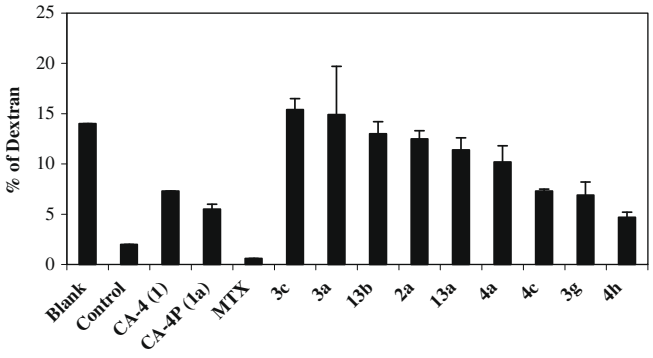


Figure 4. HUVEC permeability assay.

2.3. Conformational study

The conformational preference around the single bond joining the olefinic carbon and the carbonyl in the chalcone derivatives is an interesting and important feature that influences their activity.[38] If the system is fully conjugated, the chromophore must be planar and therefore two arrangements exist with either a C(1)–C(2)–C(3)–O(3) dihedral angle ω of 180° (the s-*trans* conformer) or 0° (the s-*cis* conformer). For electronic reasons, enone systems generally prefer to adopt an s-*trans* conformation unless forced into the s-*cis* conformation by steric hindrance (Fig. 5). A study of the conformational preference of chalcones **2a** and **3a** allowed us to speculate on the conformation required for activity.

A Monte Carlo search was performed using 1000 starting geometries. Modeling chalcone **2a** gave eight minimum energy conformations, most of which were arranged s-*cis*. The difference in energy (ΔE) between the lowest s-*cis* ($E_{s\text{-}cis}$ = 63.64 kJ mol^{-1}) and s-*trans* ($E_{s\text{-}trans}$ = 67.51 kJ mol^{-1}) conformers was small (3.87 kJ mol^{-1}). For the α-methyl substituted chalcone **3a**, all minimum energy conformers were s-*trans*. No s-*cis* conformation was found within 10 kJ mol^{-1} ($E_{s\text{-}trans}$ = 63.17 kJ mol^{-1}). We therefore conclude that the molecule **3a** prefers to adopt the s-*trans* conformation. The energy differences correspond to an s-*cis*:s-*trans* occupancy ratio of 20:40 for **2a** and >98:2 for the α-methyl chalcone **3a**. This suggests that an s-*trans* conformation is required for good biological activity.

The two rings of chalcone **3a** superimpose on those of CA4 **1** better than those of than chalcone **2a** (Fig. 6). It is tempting to speculate that the s-*trans* chalcone **3a** fits better into the binding site than the s-*cis* conformer of chalcone **2a** since the α-methyl chalcone **3a** is the most active. We therefore concluded that the conformation required for activity is the s-*trans* and later confirmed it with a QSAR study.[18] In 2004, a 3.5 Å X-ray diffraction map of thiocolchicine bound to α,β-tubulin was reported by Knossow and co-workers.[39] The colchicine derivative binds to tubulin in

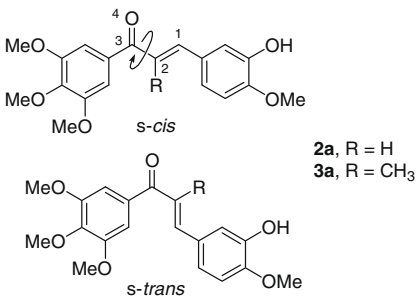


Figure 5. Conformational preference of chalcones.

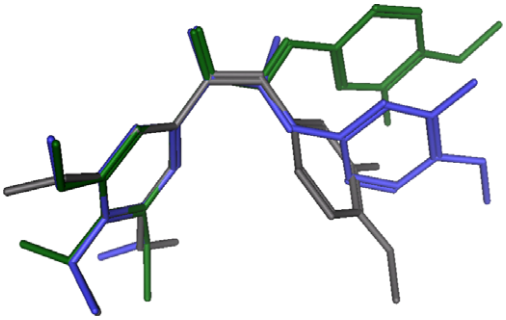


Figure 6. Superposition of CA4 (gray), **2a** (green), and **3a** (blue).

a conformation very similar to that in its native X-ray structure.[40,41] The s-*trans* conformer is similar to this X-ray structure. A more detailed study of how the agents bind to the new colchicine-binding site is reported in the accompanying paper.

3. Conclusions

The results presented in Table 1 reveal that the 3,4,5-trimethoxy A-ring substitution leads to markedly high cytotoxicity. It is notable that the most cytotoxic analogues in the series are those chalcones most resembling CA4 itself. The chalcones **2a** bearing both the A- and B-ring substitution of CA4 is the most potent. The analogue **2b** resembling 3-deoxy-amino CA4[42] is also significantly potent.

Incorporation of an alkyl group at the α-position leads to an increase in cytotoxic activity. The cytotoxic activity of **3a** is 10-fold greater than **2a**. On average the α-methyl group gives a 40-fold increase in activity where a comparison can be made (Table 2). The presence of a group at the α-position appears to act as a conformational lock forcing the chalcones to adopt an s-*trans* conformation. The cytotoxicity generally correlates with the ability of the agents to bind to tubulin at the colchicine site. Several agents have shown remarkable activity in the HUVEC shape change assay. This is a good indication that antivascular activity will be observed in vivo. The study has revealed several chalcones with high in vitro activity. The prodrugs **13a** and **13b** represent two potentially useful anticancer drugs that are currently undergoing preclinical evaluation.

4. Experimental section

4.1. Chemistry

Two-hundred megahertz ^{1}H NMR spectra were recorded using a Bruker AC 200 NMR spectrometer while all 300 MHz ^{1}H and 75 MHz ^{13}C NMR spectra were recorded using a Bruker AC 300 Spectrometer. ^{13}C NMR spectra were recorded using Distortionless Enhancement by Polarization Transfer. Both ^{1}H and ^{13}C spectra were recorded using $CDCl_3$ as internal standard. Chemical ionization (CI) mass spectra were recorded using a Kratos MS25 mass spectrometer; fast atom bombardment (FAB) mass spectra were recorded with a Kratos MS50 with a *meta*-nitrobenzyl alcohol matrix. Accurate mass determinations were carried out on a Kratos Concept IS spectrometer. Elemental analyses were performed using a Carlo-Erba 1106 elemental analyzer. Infrared spectra were recorded using a Perkin–Elmer 783 spectrometer equipped with a PE 600 data station. Melting points were determined using an Electrothermal melting point apparatus and were uncorrected. Column chromatography was conducted using Silica Gel 60 230–400 mesh (Merck & Co.). Silica TLC was conducted on pre-coated aluminum sheets (60 F_{254}) with a 0.2 mm thickness (Aldrich Chemical Co.). DMSO was distilled from calcium hydride and stored under nitrogen prior to use. Anhydrous methanol and DMF were obtained from Aldrich Chemical Co. and used as supplied.

4.1.1. General procedure for the preparation of chalcones 2

Sodium hydroxide (1 mL of a 50% w/v aqueous solution) was added to a stirred solution of benzaldehyde (10.0 mmol) and acetophenone (10.0 mmol) in methanol (30 mL). The mixture was stirred overnight at room temperature, acidified to pH 1 with 1 N aqueous solution of hydrochloric acid and extracted with dichloromethane (2 × 50 mL). The combined organic layers were dried over anhydrous magnesium sulfate, filtered evaporated in vacuo and purified by column chromatography on silica gel or recrystallization to afford the pure chalcone **2**.

(E)-3-(3″-Hydroxy-4″-methoxyphenyl)-1-(3′,4′,5′-trimethoxyphenyl)prop-2-en-1-one (**2a**): As a yellow powder (58%); mp 144–146 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.93 (3H, s), 3.95 (9H, s), 5.78 (1H, s), 6.87 (1H, d, *J* 8.3 Hz), 7.13 (1H, dd, *J* 2.0, 8.3 Hz), 7.25–7.30 (3H, m), 7.35 (1H, d, *J* 15.5 Hz), 7.75 (1H, d, *J* 15.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) 56.0, 56.4, 61.0, 106.1, 112.4, 120.4, 121.9, 128.1, 128.7, 133.6, 140.0, 142.4, 143.8, 153.0, 153.1, 189.2; λ_{max} (EtOH) 210.2 (log ε 4.62), 258.5 (log ε 3.96), and 365.0 nm (log ε 4.35); LRMS (FAB) 345 [(M+H)$^+$, 100%], 344 (M$^+$, 50). Anal. ($C_{19}H_{20}O_6$) C, H.

(E)-3-(4″-Methoxy-3″-nitrophenyl)-1-(3′,4′,5′-trimethoxyphenyl)prop-2-en-1-one (**2gg**): As a pale orange solid (61%); mp 143–145 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.95 (3H, s), 3.97 (6H, s), 4.02 (3H, s), 7.14 (1H, d, *J* 8.7 Hz), 7.29 (2H, s), 7.45 (1H, d, *J* 15.5 Hz), 7.75 (1H, d, *J* 15.5 Hz), 7.79 (1H, dd, *J* 8.7 and 2.3 Hz), 8.17 (1H, d, *J* 2.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) 56.8, 57.2, 61.4, 106, 114.2, 122.3, 125.1, 128.0, 133.5, 134.9, 140.3, 142.0, 143.2, 153.6, 154.5, 188.8; LRMS (FAB) 374 ([M+H]$^+$, 100%). Anal. ($C_{19}H_{19}NO_7$) C, H, N.

(E)-3-(3″-Amino-4″-methoxyphenyl)-1-(3′,4′,5′-trimethoxyphenyl)prop-2-en-1-one (**2b**): A mixture of (*E*)-chalcone **2gg** (1.00 g, 2.7 mmol), tin(II) chloride dihydrate (3.02 g, 13.4 mmol), and concentrated hydrochloric acid (10 drops) in 1:1 ethanol:ethyl acetate (20 mL) was stirred and heated to reflux for 2 days. The cooled mixture was diluted with ethyl acetate (30 mL) and washed with saturated sodium hydrogen carbonate solution (20 mL) followed by brine (20 mL). The organic layer was separated, dried ($MgSO_4$), and concentrated in vacuo. Purification by column chromatography (SiO_2, chloroform:ethyl acetate 4:1) afforded the chalcone **2b** as an orange-yellow solid (0.29 g, 32%), mp 90–91 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.92 (3H, s), 3.95 (3H, s), 3.96 (6H, s), 6.82 (1H, d, *J* 7.9 Hz), 7.04 (1H, s), 7.07 (1H, d, *J* 7.9 Hz), 7.28 (2H, s), 7.31 (1H, d, *J* 15.5 Hz), 7.73 (1H, d, *J* 15.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.0, 56.8, 61.4, 106.4, 110.6, 113.7, 119.7, 121.4, 128.4, 134.4, 136.9, 142.6, 145.7, 150.1, 153.5, 189.9; LRMS (EI) 343 ([M]$^+$, 100%). Anal. ($C_{19}H_{21}NO_5$) C, H, N.

(E)-3-(3″-Hydroxy-4″-methoxyphenyl)-1-(2′,3′,4′-trimethoxyphenyl)prop-2-en-1-one (**2c**): As a yellow solid (66%); mp 85–86 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.90 (12H, s), 5.73 (1H, s), 6.74 (1H, d, *J* 8.8 Hz), 6.86 (1H, d, *J* 8.1 Hz), 7.10 (1H, dd, *J* 8.1 and 2.1 Hz), 7.26 (1H, d, *J* 2.1 Hz), 7.36 (1H, d, *J* 15.8 Hz), 7.38 (1H, d, *J* 8.8 Hz), 8.61 (1H, d, *J* 15.8 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.4, 56.5, 61.4, 62.4, 107.7, 111.0, 113.5, 122.8, 125.3, 126.1, 127.4, 129.2, 142.6, 143.5, 146.3, 149.0, 154.1, 157.3, 191.3; LRMS (FAB) 244 (M$^+$, 65%), 137 (100). Anal. ($C_{19}H_{20}O_6$) C, H.

(E)-3-(3″,4″-Ethylenedioxyphenyl)-1-(3′,4′,5′-trimethoxyphenyl)prop-2-en-1-one (**2e**): A yellow powder (94%); mp 112–114 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.01 (9H, m), 4.39 (4H, m), 6.98 (1H, d, *J* 8.2 Hz), 7.22 (1H, dd, *J* 8.2 and 2.0 Hz), 7.24 (1H, d, *J* 2.0 Hz), 7.26 (2H, s), 7.40 (1H, d, *J* 15.4 Hz), 7.80 (1H, d, *J* 15.4 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.1, 60.9, 64.1, 64.6, 105.5, 117.2, 117.9,

120.9, 122.5, 130.5, 131.9, 143.4, 144.1, 147.81, 153.3, 188.9; ν_{max} (KBr disk) 1690 (C=O, s), 1600 (C=C, br), 1240 (C–O, br) cm^{-1}; LRMS (FAB) 357 [$(M+H)^+$, 100%], 187 (60). Anal. ($C_{20}H_{20}O_6$) C, H.

4.1.2. Synthesis of α-alkyl chalcones 3

4.1.2.1. General procedure for the synthesis of alkyl (3,4,5-trimethoxyphenyl)carbinols. Magnesium turnings (1.46 g, 60.1 mmol) were activated by stirring under an atmosphere of nitrogen overnight. Dry tetrahydrofuran (30 mL) was added and a solution of alkyl bromide (61.6 mmol) in dry tetrahydrofuran (15 mL) carefully added over 30 min, a under an atmosphere of nitrogen. The mixture was heated under reflux for 1 h by which time all the magnesium had dissolved. The reaction mixture was then cooled to −5 °C, before a solution of benzaldehyde (51.0 mmol) in dry tetrahydrofuran (50 mL) was added over 30 min while keeping the temperature under 0 °C. After heating the mixture under reflux overnight, a gray solution was obtained that was then quenched by adding ice (50 g) and 1 N hydrochloric acid (20 mL) dropwise. The aqueous mixture was then extracted with diethyl ether (2 × 150 mL), the combined ethereal layers were dried ($MgSO_4$), filtered and evaporated in vacuo to give the following alcohols.

1-(3′,4′,5′-Trimethoxyphenyl)propan-1-ol:[43] As a yellow liquid (98%); ^{1}H NMR ($CDCl_3$) δ 0.85 (3H, t, *J* 7.5 Hz), 1.76 (2H, m), 2.24 (1H, d, *J* 1.5 Hz), 3.84 (3H, s), 3.86 (6H, s), 4.52 (1H, td, *J* 1.5, 7.5 Hz), 6.56 (2H, s); LRMS (FAB) 227 [$(M+H)^+$, 40%], 226 (M^+, 90), 209 (100).

1-(2′,5′-Dimethoxyphenyl)propan-1-ol:[44] As a white solid (47%); mp 56–57 °C (lit mp 56–57 °C); ^{1}H NMR ($CDCl_3$) δ 1.00 (3H, t, *J* 7 Hz), 1.80 (2H, m), 2.57 (1H, d, *J* 6.6 Hz), 3.78 (3H, s), 3.82 (3H, s), 4.76 (1H, m), 6.75 (1H, dd, *J* 8.7 and 2.8 Hz), 6.82 (1H, d, *J* 8.7 Hz), 6.90 (1H, d, *J* 2.8 Hz).

1-(3′,4′,5′-Trimethoxyphenyl)pentan-1-ol:[45] As a brown oil (86%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89 (3H, t, *J* 7.1 Hz), 1.28–1.38 (4H, m), 1.65–1.75 (2H, m), 3.82 (3H, s), 3.85 (6H, s), 4.53–4.63 (1H, m), 6.55 (2H, s).

4.1.2.2. General procedure for the oxidation of the alcohols. To a stirred solution of dry DMSO (8.5 mL, 119.8 mmol) in dry dichloromethane (50 mL) at −78 °C was added, over 30 min, oxalyl chloride (5.2 mL, 59.6 mmol). The solution was stirred for 15 min at −78 °C until the evolution of gas stopped, then a solution of the alcohol (53.0 mmol) in dry dichloromethane (50 mL) was added over 30 min. The cloudy mixture was stirred at −78 °C for a further 30 min before triethylamine (37 mL, 265.4 mmol) was added over 10 min. The mixture was stirred at −78 °C for a further 5 min, allowed to warm to room temperature and stirred for 2 h. The mixture was then poured into chloroform (200 mL), the organic layer washed with 1 N hydrochloric acid (50 mL) and water (100 mL) and dried ($MgSO_4$), filtered and evaporated in vacuo. The crude solid residue was recrystallized from hexane/chloroform (5:1) to give the following ketones.

1-(3′,4′,5′-Trimethoxyphenyl)propan-1-one:[46] As pale yellow needles (90%); mp 54–56 °C (lit. mp 52–53 °C); ^{1}H NMR ($CDCl_3$) δ 1.23 (3H, t, *J* 7.3 Hz), 2.98 (2H, q, *J* 7.3 Hz), 3.92 (9H, s), 7.23 (2H, s).

1-(2′,5′-Dimethoxyphenyl)propan-1-one: As a pale yellow oil (84%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.09 (3H, t, *J* 7.3 Hz), 2.93 (2H, q, *J* 7.3 Hz), 3.71 (3H, s), 3.79 (3H, s), 6.89 (1H, d, *J* 8.5 Hz), 7.23 (1H, d, *J* 2.9 Hz), 7.99 (1H, dd, *J* 8.5 and 2.9 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 8.4, 37.0, 55.7, 56.0, 112.6, 114.0, 119.4, 125.0, 153.1, 153.5, 202.9; LRMS (FAB) 195 [$(M+H)^+$, 95%], 194 [M^+, 95], 165 (100). Anal. ($C_{11}H_{14}O_3$) C, H.

1-(3′,4′,5′-Trimethoxyphenyl)pentan-1-one: As a yellow oil (81%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.95 (3H, t, *J* 7.4 Hz), 1.40 (2H, sextet, *J* 7.4 Hz), 1.70 (2H, pentet, *J* 7.4 Hz), 2.92 (2H, t, *J* 7.4 Hz), 3.90 (9H, s), 7.23 (2H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.9, 20.9, 25.5, 37.9, 56.2, 60.8, 105.0, 132.3, 142.3, 150.0, 199.2; LRMS (FAB) 253 [$(M+H)^+$, 100%], 195 (80). Anal. ($C_{14}H_{20}O_4$) C, H.

4.1.2.3. General Procedure for the preparation of the chalcones 3. A mixture of ketone (45.2 mmol), benzaldehyde (44.1 mmol), piperidine (10 mL), and glacial acetic acid (5 mL) in dry ethanol (50 mL) was heated under reflux and water was removed from the reaction mixture by Soxhlet extraction over 4 Å molecular sieves for 2 days. The solvent was removed in vacuo and the residue was purified by flash column chromatography on silica to give the α-alkyl chalcone **3**. These chalcones were exclusively isolated as *E*-isomers as reported previously.[24]

(E)-3-(3″-Hydroxy-4″-methoxyphenyl)-2-methyl-1-(3′,4′,5′-trimethoxyphenyl)prop-2-en-1-one (**3a**): As a white solid (20%). Mp 112–114 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.28 (3H, s), 3.89 (6H, s), 3.93 (6H, s), 5.70 (1H, s), 6.89 (1H, d, *J* 8.3 Hz), 6.95 (1H, dd, *J* 8.3 and 2.0 Hz), 7.00 (2H, s), 7.10 (1H, d, *J* 2.0 Hz), 7.11 (1H, s); ^{13}C NMR (75 MHz, $CDCl_3$) 15.1, 56.4, 56.7, 61.3, 107.5, 110.8, 116.1, 123.2, 129.6, 134.1, 135.4, 141.6, 141.9, 145.9, 147.4, 153.2, 199.0; LRMS (FAB) 359 [$(M+H)^+$, 100%]. Anal. ($C_{20}H_{22}O_6$) C, H.

(E)-3-(3″-Hydroxy-4″-methoxyphenyl)-2-propyl-1-(3′,4′,5′-trimethoxyphenyl)prop-2-en-1-one (**3b**): As a white solid (44%). Mp 96–97 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.01 (3H, t, *J* 7.5 Hz), 1.57 (2H, sextet, *J* 7.5 Hz), 2.72 (2H, t, *J* 7.5 Hz), 3.88 (6H, s), 3.92 (6H, s), 5.70 (1H, s), 6.88 (1H, d, *J* 2.8 Hz), 6.98 (1H, s), 7.00–7.02 (2H, m), 7.03 (2H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.7, 22.4, 30.3, 56.4, 56.7, 61.3, 107.6, 110.9, 115.7, 122.5, 129.4, 134.2, 140.4, 140.9, 141.9, 145.9, 147.3, 153.2, 198.9; LRMS (FAB) 387 [$(M+H)^+$, 100%], 195 (70). Anal. ($C_{22}H_{26}O_6$) C, H.

(E)-3-(3″-Fluoro-4″-methoxyphenyl)-2-methyl-1-(3′,4′,5′-trimethoxyphenyl)prop-2-en-1-one (**3c**)*:* As a white solid (62%). Mp 96–97 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.26 (3H, s), 3.89 (6H, s), 3.92 (6H, s), 6.98 (2H, s), 6.99 (1H, t, *J* 8.6 Hz), 7.08 (1H, s), 7.17 (1H, dd, *J* 10.4 and 1.9 Hz), 7.24 (1H, ddd, *J* Hz, 8.6, 1.9, and 1.6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.1, 56.6, 56.7, 61.3, 107.5, 113.5 (d, *J* 2.0 Hz), 117.6 (d, *J* 15.0 Hz), 127.0 (d, *J* 5.0 Hz), 129.2 (d, *J* 5.0 Hz), 136.1, 133.8, 140.3, 141.8, 148.4 (d, *J* 15.0 Hz), 152.4 (d, *J* 247.0 Hz), 153.2, 198.7; δ_F (188 MHz, $CDCl_3$) δ −56.8 (m); LRMS (FAB) 361 [$(M+H)^+$, 100%], 191 (80). Anal. ($C_{20}H_{21}O_5F$) C, H, F.

(E)-3-(3″,4″-Ethylenedioxyphenyl)-2-methyl-1-(3′,4′,5′-trimethoxyphenyl)prop-2-en-1-one (**3d**)*:* As a white powder (94%); mp 125–127 °C; ^{1}H NMR ($CDCl_3$) δ 2.24 (3H, d, *J* 1.3 Hz), 3.90 (6H, s), 3.93 (3H, s), 4.30 (4H, br s), 6.89–6.98 (2H, m), 6.99 (2H, s), 7.02 (1H, d, *J* 1.9 Hz), 7.10 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.2, 56.0, 60.6, 64.0, 64.2, 106.8, 117.1, 118.4, 123.5, 129.4, 133.4, 134.6, 140.9, 141.1, 143.1, 144.0, 152.5, 198.1; LRMS (FAB) 371 [$(M+H)^+$, 100%], 370 (M^+, 90). Anal. ($C_{21}H_{22}O_6$) C, H.

4.1.3. Synthesis of α-alkoxy chalcones 4

2-Bromo-1-(3′,4′,5′-trimethoxyphenylethan-1-one:[47] To a stirring solution of 3,4,5-trimethoxyacetophenone (47.6 mmol) in dry diethyl ether (450 mL) at 0 °C under argon was added bromine (2.70 mL, 52.3 mmol) in dry ether (250 mL). On completion of addition the flask was irradiated with a 125 W light source for 1 h. The mixture was washed with an aqueous solution (saturated) of sodium metabisulfite (2 × 200 mL) and the organic fraction dried over anhydrous magnesium sulfate, filtered and evaporated in vacuo. Recrystallization afforded 2-bromo-1-(3′,4′,5′-trimethoxyphenylethan-1-one as a white solid (85%). Mp 64–66 °C [lit. mp 63–67 °C]; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.94 (9H, s), 4.41 (2H, s), 7.22 (2H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 30.6, 56.4, 61.1, 106.6, 129.0, 143.4, 153.2, 190.3; LRMS (FAB) 291 [$(M+H)^+$, ^{81}Br, 40%], 289 [$(M+H)^+$, ^{79}Br, 45], 195 (100). Anal. ($C_{11}H_{13}O_4Br$) C, H.

4.1.3.1. General procedure for the preparation of α-alkoxyacetophenones. To a stirring solution of 2-bromo-1-(3′,4′,5′-trimeth-

oxyphenylethan-1-one (14.5 mmol) in alcohol (ROH where R = Me, Et, Pr, 40 mL) was added silver carbonate (5.00 g, 18.2 mmol) and boron trifluoride etherate (2.10 mL, 16.7 mmol). The solution was stirred at room temperature under argon for 2 days, filtered, diluted with dichloromethane (100 mL), washed with water (50 mL) and the organic fraction dried over anhydrous magnesium sulfate, filtered and evaporated in vacuo. The crude residue was purified by flash column chromatography on silica to provide the following α-alkoxyacetophenones.

2-Methoxy-1-(3′,4′,5′-trimethoxyphenyl)-1-ethanone:[48] As a white solid (74%); mp 54–55 °C [lit. mp 54 °C]; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.51 (3H, s), 3.93 (9H, s), 4.68 (2H, s), 7.20 (2H, s).

2-Ethoxy-1-(3′,4′,5′-trimethoxyphenyl)-1-ethanone: As a pale yellow oil (91%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.28 (3H, t, *J* 7.0 Hz), 3.63 (2H, q, *J* 7.0 Hz), 3.90 (9H, s), 4.68 (2H, s), 7.22 (2H, s, H-2′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.5, 56.7, 61.3, 67.6, 74.1, 105.9, 130.5, 143.3, 153.5, 195.8; LRMS (FAB) 255 [(M+H)$^+$, 100%]; HRMS calcd 254.1153; found 354.1154 for $C_{13}H_{18}O_5$ for M$^+$.

2-Propoxy-1-(3′,4′,5′-trimethoxyphenyl)-1-ethanone: As a colorless oil (62%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (3H, t, *J* 7.2 Hz), 1.68 (2H, sextet, *J* 7.2 Hz), 3.53 (2H, t, *J* 7.2 Hz), 3.91 (9H, s), 4.68 (2H, s), 7.25 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.9, 23.3, 56.7, 61.4, 73.9, 74.4, 106.0, 130.6, 143.3, 153.5, 196.0; LRMS (FAB) 269 [(M+H)$^+$, 70%], 195 (100). Anal. ($C_{14}H_{20}O_5$) C, H.

4.1.3.2. General procedure for the preparation of the α-alkoxychalcones 4. The following chalcones were prepared using the same procedure used to make the α-alkylchalcones of type **3**.

(Z)-3-(3″-Hydroxy-4″-methoxyphenyl)-2-methoxy-1-(3′,4′,5′-time thoxyphenyl)prop-2-en-1-one (**4a**): As a yellow solid (31%). Mp 120–122 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.77 (3H, s), 3.91 (6H, s), 3.93 (3H, s), 3.94 (3H, s), 5.62 (1H, s), 6.85 (1H, d, *J* 8.6 Hz), 6.46 (1H, s), 7.18 (2H, s), 7.21 (1H, dd, *J* 8.6 and 2.1 Hz), 7.53 (1H, d, *J* 2.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.3, 56.7, 58.9, 61.3, 107.5, 110.8, 116.3, 123.7, 124.6, 127.8, 133.2, 142.6, 145.8, 147.7, 152.5, 153.4, 192.0; LRMS (FAB) 374 [M$^+$, 100%], 195 (100). Anal. ($C_{20}H_{22}O_7$) C, H.

(Z)-3-(3″-Fluoro-4″-methoxyphenyl)-2-methoxy-1-(3′,4′,5′-trimethoxyphenyl)prop-2-en-1-one (**4c**): As a yellow solid (62%). Mp 110–112 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.78 (3H, s), 3.92 (3H, s), 3.93 (6H, s), 3.95 (3H, s), 6.41 (1H, s), 6.95 (1H, t, *J* 8.6 Hz), 7.19 (2H, s), 7.37 (1H, dd, *J* 10.1 and 2.0 Hz), 7.74 (1H, ddd, *J* 8.6, 2.0, and 1.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 56.6, 56.8, 59.0, 61.4, 107.4, 113.2 (d, *J* 3.0 Hz), 117.6 (d, *J* 15.0 Hz), 122.8 (d, *J* 3.0 Hz), 127.3 (d, *J* 6.0 Hz), 127.5 (d, *J* 6.0 Hz), 132.9, 142.7, 148.6 (d, *J* 15.0 Hz), 152.4 (d, *J* 245.0 Hz), 152.9, 153.4, 191.7 (C); ^{19}F NMR (188 MHz, $CDCl_3$) δ −57.0 (m); LRMS (FAB) 377 [(M+H)$^+$, 100%]. Anal. ($C_{20}H_{21}O_6F$) C, H.

(Z)-2-Ethoxy-3-(3″-fluoro-4″-methoxyphenyl)-1-(3′,4′,5′-trimetho xyphenyl)prop-2-en-1-one (**4d**): As a yellow solid (54%); mp 89–90 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.38 (3H, t, *J* 7.0 Hz), 3.92 (6H, s), 3.93 (3H, s), 3.95 (3H, s), 3.99 (2H, q, *J* 7.0 Hz), 6.43 (1H, s), 6.95 (1H, t, *J* 8.8 Hz), 7.22 (2H, s, H-2), 7.40 (1H, dd, *J* 10.1 and 1.9 Hz), 7.80 (1H, ddd, *J* 8.8, 1.9, and 1.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) 16.0, 56.6, 56.7, 61.4, 67.4, 107.4, 113.3 (d, *J* 3.0 Hz), 117.6 (d, *J* 15.0 Hz), 122.6 (d, *J* 3.0 Hz), 127.2 (d, *J* 6.0 Hz), 127.7 (d, *J* 6.0 Hz), 132.7, 142.8, 148.5 (d, *J* 15.0 Hz), 152.1, 152.4 (d, *J* 245.0 Hz), 153.3, 191.9; ^{19}F NMR (188 MHz, $CDCl_3$) δ −55.6 (m); LRMS (FAB) 391 [(M+H)$^+$, 100%]. Anal. ($C_{21}H_{23}O_6F$) C, H.

4.1.3.3. General synthesis of the chalcone epoxides 5. A mixture of chalcone **2** (10.0 mmol), *t*-butyl hydroperoxide (70% aqueous solution, 1.5 mL, 10.9 mmol), and Triton B® (40% methanolic solution of benzyltrimethylammonium hydroxide, 0.25 mL, 0.55 mmol) in methanol (20 mL) was stirred at room temperature under an atmosphere of nitrogen overnight. The resulting precipitate was collected, washed with cold methanol and recrystallized from methanol to give the pure epoxide **5**.

(2RS,3SR)-2,3-Epoxy-3-(4″-methylphenyl)-1-(3′,4′,5′-trimethoxyp henyl)propan-1-one (**5a**): As a white powder (83%); mp 128–130 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.43 (3H, s), 3.86 (3H, s), 3.87 (6H, s), 4.02 (1H, d, *J* 1.8 Hz), 4.23 (1H, d, *J* 1.8 Hz), 6.58 (2H, s), 7.29 (2H, d, *J* 8.2 Hz), 7.92 (2H, d, *J* 8.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.7, 56.1, 59.4, 60.7, 60.8, 102.9, 128.4, 129.5, 131.2, 133.0, 138.4, 145.0, 153.6, 192.3; λ_{max} (EtOH) 207.0 (log ε 4.76) and 261.9 nm (log ε 4.27); LRMS (FAB) 329 [(M+H)$^+$, 50%], 328 (M$^+$, 60), 181 (50), 154 (40), 119 (100). Anal. ($C_{19}H_{20}O_5$) C, H.

4.1.4. Reduction of chalcones

(E)-3-(3″-Hydroxy-4″-methoxyphenyl)-2-methyl-1-(3′,4′,5′-trimethoxyphenyl)prop-2-en-1-ol (**6a**): A mixture of (*E*)-chalcone **3a** (0.30 g, 0.84 mmol), sodium borohydride (0.06 g, 1.70 mmol), and cerium(III) chloride heptahydrate (0.31 g, 0.84 mmol) in methanol (20 cm^3) and water (1 mL) was stirred at 0 °C for 1 h. The subsequent mixture was diluted with water (20 cm^3) and extracted with chloroform (2 × 30 mL). The organic layer was washed with saturated aqueous ammonium chloride solution (30 mL), separated, dried ($MgSO_4$), and evaporated in vacuo to give the alcohol **6a** as a pale yellow solid (0.30 g, 99%) mp 100–102 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.79 (3H, s), 2.03 (1H, s, OH), 3.88 (3H, s), 3.89 (6H, s), 3.93 (3H, s), 5.23 (1H, s), 5.63 (1H, s), 6.68–6.73 (3H, m), 6.84–6.88 (2H, m), 6.96 (1H, d, *J* 1.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 56.4, 56.5, 61.2, 80.0, 103.8, 110.8, 115.5, 121.5, 126.2, 131.3, 137.6, 138.2, 138.6, 145.6, 145.8, 153.6; LRMS (FAB) 360 (M$^+$, 100%). Anal. ($C_{20}H_{24}O_6$) C, H.

3-(3″-Hydroxy-4″-methoxyphenyl)-2-methyl-1-(3′,4′,5′-trimethoxyphenyl)propan-1-one (**8a**): (*E*)-Chalcone **3a** (0.3 g, 0.84 mmol) in ethyl acetate (10 mL) as added to a suspension of activated 10% Pd/C (1 spatula) in ethyl acetate (10 mL) under a hydrogen atmosphere. The mixture was stirred at room temperature for 2 h and then filtered through celite and evaporated in vacuo. Purification by column chromatography (SiO_2, hexane:ethyl acetate 3:2) afforded the ketone **8a** as a beige solid (0.17 g, 57%); mp 113–114 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (3H, d, *J* 6.8 Hz), 2.63 (1H, dd, *J* 13.9 and 7.2 Hz), 3.05 (1H, dd, *J* 13.9 and 6.8 Hz), 3.66 (1H, m), 3.86 (3H, s), 3.90 (6H, s), 3.92 (3H, s), 5.59 (1H, s), 6.67 (1H, dd, *J* 8.3 and 1.9 Hz), 6.75 (1H, d, *J* 8.3 Hz), 6.81 (1H, d, *J* 1.9 Hz), 7.16 (2H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 18.1, 39.7, 43.1, 56.3, 56.6, 61.3, 106.2, 110.9, 115.4, 121.0, 132.3, 133.6, 142.8, 145.4, 145.9, 153.4, 203.2; LRMS (FAB) 360 (M$^+$, 28%), 137 (100); Anal. Calcd ($C_{20}H_{24}O_6$), C, H. Further elution of the column gave 3-(3″-hydroxy-4″-methoxy-phenyl)-2-methyl-1-(3′,4′,5′-trimethoxyphenyl)propan-1-ol (**7a**) as a clear liquid (0.13 g, 44%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.71 and 0.89 (3H, d, *J* 6.8 Hz), 1.86 and 1.95 (1H, s, OH), 2.00–2.06 and 2.07–2.14 (1H, m), 2.37–2.34 and 2.35–2.41 (1H, m), 2.66–2.72 and 2.94–3.00 (1H, m), 3.86 and 3.86 (3H, s), 3.88 and 3.88 (6H, s), 3.89 and 3.89 (3H, s), 4.41 and 4.52 (1H, d, *J* 4.9 Hz), 5.59 and 5.59 (1H, s), 6.56 and 6.58 (2H, s), 6.65 and 6.69 (1H, dd, *J* 8.3 and 2.3 Hz), 6.78 and 6.79 (1H, d, *J* 8.3 Hz), 6.76 and 6.81 (1H, d, *J* 2.3 Hz); LRMS (FAB) 362 (M$^+$, 54%), 41 (100). HRMS calcd 362.1729; found 362.1729 for $C_{20}H_{26}O_6$ (M$^+$).

2-(4″-Methoxyphenyl)-1-(3′,4′,5′-trimethoxyphenyl)propane (**9a**):[49] (*E*)-Chalcone **2ii** (5.0 g, 15.2 mmol) in dichloromethane (50 mL) was added to a stirring activated suspension of 10% Pd/C (1 spatula) in dichloromethane (10 mL) under a hydrogen atmosphere. The mixture was stirred at room temperature for 30 min, filtered through celite and evaporated in vacuo. Purification by column chromatography (SiO_2, hexane:chloroform 1:1, long column) afforded the diarylpropane **9a** as an off-white solid (1.6 g, 33%); mp 57–59 °C (lit. mp 57.5–58.5 °C); ^{1}H NMR ($CDCl_3$) δ 1.91–1.99 (2H, m), 2.59–2.66 (4H, m), 3.83 (3H, s), 3.86 (3H, s), 3.88 (6H, s), 6.42

(2H, s), 6.87 (2H, d, *J* 9.0 Hz), 7.14 (2H, d, *J* 9.0 Hz). Further elution of the column gave 1-(4″-methoxyphenyl)-3-(3′,4′,5′-trimethoxyphenyl)propan-1-one (**8d**) (2.5 g, 50%) as an off-white colored solid, mp 98–100 °C (lit.[50] mp 98 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.02 (2H, t, *J* 7.5 Hz), 3.26 (2H, t, *J* 7.5 Hz), 3.84 (3H, s), 3.86 (6H, s), 3.89 (3H, s), 6.48 (2H, s), 6.94 (2H, d, *J* 9.0 Hz), 7.79 (2H, d, *J* 9.0 Hz).

4.1.5. Synthesis of phosphate derivatives 12 and 13

4.1.5.1. Disodium 3′-phosphate salt 13a. According to the method of Perich and Jones,[51] 1*H*-tetrazole (408 mg, 5.82 mmol) was added in one portion to a stirred solution of chalcone **3a** (583 mg, 1.69 mmol) and di-*tert*-butyl *N,N*-diethylphosphoramidite (0.43 mL, 1.54 mmol) in dry tetrahydrofuran (5 mL) and stirred for 20 min at room temperature under an atmosphere of nitrogen. The mixture was then cooled to −78 °C and a solution of *m*-CPBA (57% w/w, 631 mg, 2.08 mmol) in dry dichloromethane (2 mL) was added. After stirring for 10 min at room temperature, a 10% aqueous solution of sodium bisulfite (4 mL) was added and the mixture stirred for a further 15 min. The aqueous mixture was then extracted with diethyl ether (50 mL) and the ethereal layer washed with a 10% aqueous solution of sodium bisulfite (2 × 20 mL), a 5% aqueous solution of sodium bicarbonate (2 × 20 mL), a 0.5 M aqueous solution of sodium hydroxide (2 × 20 mL) and finally water (20 mL). The ethereal layer was then dried ($MgSO_4$), filtered, and evaporated in vacuo to give the phosphate **11a** (770 mg, 1.43 mmol, 85%); LRMS (FAB) 539 [$(M+H)^+$, 40%], 425 (30). A solution of 10 M hydrochloric acid:1,4-dioxane (1:1, 10 mL) was added to the residue and the reaction was allowed to stand at room temperature for 1 h. The solvent was evaporated under reduced pressure (keeping the temperature below 45 °C) and water (15 mL) was added to the residue. The resultant precipitate was collected and washed with chloroform (20 mL) to give the 3′-phosphoryl chalcone **12a** as a yellow oil (390 mg, 0.92 mmol, 54%); ^{1}H NMR (300 MHz, DMSO-d_6) 3.07 (3H, s, OMe), 3.12 (3H, s, OMe), 3.15 (6H, s, OMe), 6.33 (1H, d, *J* 8.8 Hz, H-5′), 6.61 (2H, s, H-2″, H-6″), 6.75 (1H, dd, *J* 4.4, 8.8 Hz, H-6′), 6.88–7.00 (3H, m, H-1, H-2, H-2′); ^{31}P NMR (81 MHz, DMSO-d_6) −0.17; LRMS (FAB) 425 [$(M+H)^+$, 100%], 424 (M^+, 50). Chalcone **12a** (108 mg, 0.25 mmol) was dissolved in a 1:1 mixture of methanol:water (4 mL) and two drops of a 35% w/v aqueous ammonia solution were added. According to the method of Pettit and co-workers,[29] the mixture was applied to a Dowex 50 W-X8 cation-exchange column (10 cm^3, Na^+), the column was eluted with a 1:1 mixture of methanol:water (30 mL) and the eluent was concentrated to give disodium 3′-phosphoryl chalcone **13a** as a bright yellow powder (87 mg, 0.19 mmol, 76%); mp 160 °C (dec.); λ_{max} (EtOH) 206.7 (log ε 4.41) and 358.9 nm (log ε 4.01); ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.07 (3H, s, OMe), 3.12 (3H, s, OMe), 3.15 (6H, s, OMe), 6.33 (1H, d, *J* 8.8 Hz, H-5′), 6.61 (2H, s, H-2″, H-6″), 6.75 (1H, dd, *J* 2.4, 8.8 Hz, H-6′), 6.88–7.00 (3H, m, H-1, H-2, H-2′); ^{31}P NMR (81 MHz, DMSO-d_6) δ −87.2; LRMS (FAB) 491 [$(M+Na)^+$, 60%], 469 [$(M+H)^+$, 60], 329 (50), 176 (100); HRMS calcd 469.0641; found 469.0630 for $C_{19}H_{20}O_9PNa_2$ $(M+H)^+$.

4.1.5.2. Disodium 3′-phosphate salt 13b. 1*H*-Tetrazole (237 mg, 3.38 mmol) was added to a stirred solution of chalcone **4a** (970 mg, 2.71 mmol) and di-*tert*-butyl *N,N*-diethylphosphoramidite (0.75 cm^3, 2.69 mmol) in dry dichloromethane (10 mL) and stirred for 20 min at room temperature under an atmosphere of nitrogen. The reaction mixture was then cooled down to −78 °C and *m*-CPBA (57% w/w, 945 mg, 3.12 mmol, dried over anhydrous magnesium sulfate) in dry dichloromethane (5 mL) was added. After stirring for 10 min at room temperature, a 10% aqueous solution of sodium bisulfite (8 mL) was added and the mixture was stirred for a further 15 min. The aqueous mixture was extracted with diethyl ether (30 mL) and the ethereal layer was washed successively with a 10% aqueous solution of sodium bisulfite (10 mL), a 5% aqueous solution of sodium bicarbonate (10 mL), a 0.5 M aqueous solution of sodium hydroxide (10 mL) and finally with water (10 mL). The solvent was removed in vacuo. The residue was redissolved in 10 M hydrochloric acid:1,4-dioxane (1:1, 10 mL) and then the mixture was left to stand at room temperature for 2 h. The solvents were removed and water (20 mL) was added to the residue. The resultant precipitate was collected by filtration, washed with water (20 mL) and dissolved in a 1:1 mixture of methanol:water and two drops of a 35% w/v aqueous solution of ammonia were added. The mixture was applied to a Dowex 50 W-X8 cation-exchange resin column (15 mL, Na^+), eluted with water (30 mL) and then concentrated to give disodium 3′-phosphoryl chalcone **13b** as a yellow powder (40 mg, 0.083 mmol, 39%); mp 170 °C (dec.); λ_{max} (EtOH) 208.6 (log ε 4.52) and 326.2 nm (log ε 4.12); ^{1}H NMR (300 MHz, D_2O) δ 2.20 (3H, s, Me), 3.82 (3H, s, OMe), 3.84 (6H, s, OMe), 3.86 (3H, s, OMe), 6.98 (2H, s, H-2″, H-6″), 7.02 (1H, d, *J* 8.5 Hz, H-5′), 7.14 (2H, m, H-2′, H-6′), 7.60 (1H, br s, H-2); ^{13}C NMR (75 MHz, D_2O) 15.2, 57.3, 57.6, 62.4, 99.9, 108.8, 113.7, 123.4, 126.8, 129.6, 135.9, 141.4, 144.4, 146.7, 152.4, 153.5, 204; ^{31}P NMR (81 MHz, D_2O) δ −87.0; LRMS (FAB) 505 [$(M+Na)^+$, 60%], 483 [$(M+H)^+$, 75], 391 (30), 329 (30), 289 (40), 176 (100), 136 (50); HRMS calcd 483.0798; found 483.0812 (FAB) for $C_{20}H_{22}O_9PNa_2$ $(M+H)^+$.

4.2. Biological evaluation

4.2.1. Cell inhibitory properties[29]

Cells were cultured in RMPI medium, free of antibiotics and containing 2-mercaptoethanol (2 μM) and L-glutamine (2 μM), supplemented with fetal calf serum (FCS) (10% v/v). At monthly intervals all cell lines were inspected for mycoplasma contamination by the staff of the Paterson Institute. The cells were adjusted to a concentration depending on their observed doubling time, (ca. 2000 cells/mL), in RPMI medium supplemented with FCS (10% v/v). The candidate drug was dissolved in DMSO. To 4 mL of cell solution was added 4 μL of the drug solution and 1 mL of this solution was added to 1 mL of cell solution in the adjacent tube, giving a drug concentration half that of the first dilution. This series of dilutions was continued to afford seven samples at different concentrations leaving one cell solution free of drug acting as a control. Aliquots of 200 μL of the treated cells were then pipetted in triplicate into a 96-well microtitre testplate and incubated (37 °C, 5% CO_2 in air) for 5 days. After this time, the plate was removed from the incubator and 50 μL of a solution of MTT (3 mg/mL in PBS) was added to each well. After incubation (37 °C, 5% CO_2 in air, 3 h) the medium was carefully removed from each well by suction and the resulting formazan precipitate was redissolved in 200 μL of DMSO. The optical density of each well was then read at two wavelengths (λ 540 and 690 nm) using a Titretek Multiscan MCC/340 platereader. After processing and analysis through the application of an 'in-house' software package, the results obtained enabled the calculation of the drug dose required to inhibit cell growth by 50% (IC_{50} value), determined by graphical means as a percentage of the control growth.

4.2.2. Cell cycle analysis

To K562 cells in RPMI medium supplemented with FCS (10% v/v) (10 mL, 2 × 10^5 cells/mL) was added the candidate drug (10 μL, 5000 × IC_{50} in DMSO). After incubation (37 °C, 5% CO_2 in air, overnight) the cells were centrifuged (1800 RPM, 10 min) and the supernatant discarded. The resulting pellet was re-suspended in a cold 1:1 mixture of acetone:ethanol (10 mL) and the mixture was stored at 4 °C until analysis was performed. The fixed cells were centrifuged (1800 RPM, 10 min) and the supernatant

discarded. The resulting cell pellet was re-suspended in ice cold PBS (10 mL) and centrifuged (1800 RPM, 10 min), again the supernatant discarded. The next stage involved the incubation (37 °C, 15 min) of the cells with RNAse (150 μL, 100 μg/mL in PBS, DNAse free, from bovine pancreas). The cell pellet was dissolved a solution of propidium iodide (0.25 mL, 50 μg/mL in PBS) and transferred to flow cytometry tubes before analysis by flow cytometry. The intracellular fluorescence was measured in a Coulter Epics V flow cytometer, using an argon laser (λ_{ex} 488 nm).

4.2.3. Immunohistochemistry

VERO cells (2×10^4 cells/mL), grown in semi-confluence over 2 days in an eight-well chamber slides (Nunc. Inc.), were treated with the candidate drug [200 μL, 10 μM in 1% DMSO and RPMI/FCS (10%)] and the slide incubated (37 °C, 1 h). Having removed the medium the slide was washed with PIPES buffer [2×200 μL, (0.1 M PIPES, 4% w/v PEG6000, 1 mM EDTA, 0.5 mM $MgCl_2$, distilled water, pH 6.8)] for 5 min. The slide was fixed with a solution of paraformaldehyde (200 μL, 3.5% w/v in PBS) for 10 min and washed with PIPES buffer (2×200 μL). Using a cold 1:1 mixture of acetone/ethanol (200 μL, 7 min, −20 °C) the slide was permeabilized and washed again with PIPES buffer (2×200 μL).

A solution of sodium borohydride (200 μL, 5 mg/mL in PBS, 3×5 min) was used to reduce any free aldehyde remaining and the slide washed with PBS (200 μL), before the cells were blocked with 200 μL of a solution containing bovine serum albumin (BSA, 0.1% w/v) and sodium azide (20 mM) in PBS. After incubation (37 °C, 40 min), the slide was stained with the primary antibody TAT1 (200 μL, 1/30 dilution in PBS). After a further incubation (37 °C, 70 min), the slide was washed with PBS (200 μL, 3×5 min), treated with a fluorescently labeled secondary antibody (200 μL, 1/35 dilution in PBS) and returned to the incubator (37 °C, 50 min). The slide was washed with PBS (200 μL, 2×5 min) and rinsed under the tap for another 5 min. On removing the upper structure of the slide a drop of fluorescent mounting medium was added and the cover slip was applied. The slide was placed at 0 °C in a dark place before visualization, a technique was performed under a UV microscope using a suitable filter for FITC and photographed using a 1600 ASA 35 mm film.

4.2.4. Tubulin assembly assay

The method of Woods was used in this study.[2] Six samples were prepared directly in quartz cuvettes at 0 °C and contained Mes buffer [740 μL (0.1 M Mes, 1 mM EGTA, 0.5 mM $MgCl_2$, distilled water, pH 6.6)], GTP (100 μL, 10 mM in Mes buffer), tubulin (150 μL, 10–15 μM in Mes buffer), and the candidate drug (10 μL, decreasing concentration starting at 10 mM in DMSO). The tubulin/drug samples were immediately placed in a Varian Cary 1 UV/visible spectrophotometer, preheated at 37 °C, alongside six blank samples containing Mes buffer (900 μL) and GTP (100 μL). Recording the absorbance (λ 350 nm) for a period of 20 min, the results were compared to the untreated control cells to evaluate the relative degree of change in optical density. Blank samples containing the drug candidate in Mes buffer were recorded in parallel to ascertain that no artefact reaction was occurring between the drug and the chemicals in the buffer.

4.2.5. Colchicine competition assay

The method of Na and Timasheff was applied.[52] Six samples were prepared (in triplicate) directly in Eppendorf vials and contained Mes buffer (0.1 M Mes, 1 mM EDTA, 1 mM EGTA, 1 mM magnesium chloride, 1 mM β-mercaptoethanol, distilled water, pH 6.4), colchicine (10 μM in Mes buffer plus 0.1% [^{3}H]colchicine (~60 Ci/mmol, 1 mCi/mL)), tubulin (10 μM in Mes buffer) and the candidate drug (10 mM (A), 1 mM (B), 0.1 mM (C) 0.05 mM (D) in DMSO). After incubation in the dark (rt, 2 h), the mixtures were cooled on ice for 5 min. The samples (150 μL) were transferred in triplicate to the top of Sephadex columns [prepared from 1 mL syringes, glass wool, and 1 mL Sephadex G-50 pre-swollen in Mes buffer and centrifuged (900 RPM, 2 min) then transferred to a clean tube and centrifuged (900 RPM, 2 min). The eluent (100 μL) was added to the scintillant liquid (5 mL, Ecoscint A, Mensura Ltd) and the levels of radioactivity were measured by scintillation spectrometry (Beckman LS 1801). When tubulin was not present in the reaction mixture the level of [^{3}H]colchicine was negligible, having remained bound to the column.

4.2.6. HUVEC permeability assay

The HUVEC permeability assay was carried out by Dr. Tim Ward of the Paterson Institute for Cancer Research. The cell line employed in this technique are Human Umbilical Vascular Epithelial Cells (HUVEC). The cells are grown on a porous filter within a hollow chamber, forming a confluent layer across the permeable surface. The cells are cultured in RPMI medium, free of antibiotics, containing 2-mercaptoethanol (2 μM) and L-glutamine (2 μM), supplemented with 10% (v/v) fetal calf serum (FCS). To attain a good surface coverage of cells, incubation at 37 °C for 48 h in an atmosphere of 5% carbon dioxide in air, prior to treatment is recommended. The cell chamber is transferred to a well containing a drug solution at a concentration of 1 μM in RPMI. The plate is then incubated at 37 °C for 1 h, in an atmosphere of 5% carbon dioxide in air. After 1 h the cell chamber is treated with a fluorescent-labeled high molecular weight dextran (F-Dextran), with a further 30 min incubation to allow time for the marker to pass through the membrane. The quantity of the dextran collected upon passage through the disk, its rate of flow, relates to the extent of the surface area covered by the cells on the disk.

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Supplementary data

Supplementary data (supplementary data for the following compounds are provided. Chalcones **2d**, **2f**–**ε**, **3e**–**l**, **4e–h, 5b–h, 6b–c, 7b–c, 8b–c**. Microanalytical data are also provided) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.09.039.

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