Synthesis of a Natural Chalcone and Its Prenyl Analogs – Evaluation of Tumor Cell Growth-Inhibitory Activities, and Effects on Cell Cycle and Apoptosis

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Six prenyl (= 3-methylbut-2-en-1-yl) chalcones (= 1,3-diphenylprop-2-en-1-ones), **2**–**7**, and one natural non-prenylated chalcone, **1**, have been synthesized and evaluated for their *in vitro* growth-inhibitory activity against three human tumor cell lines. A pronounced dose-dependent growth-inhibitory effect was observed for all prenylated derivatives, except for **7**. The chalcone possessing one prenyloxy group at C(2′), *i.e.*, **2**, was the most active derivative against the three human tumor cell lines ($5.9 < GI_{50} < 7.7$ μM). The majority of compounds caused an increase in percentage of apoptotic cells and/or they interfered with cell cycle distribution in the MCF-7 cell line.

Introduction. – Flavonoids represent an outstanding class of naturally occurring compounds with interesting biological activities, with the antitumor effect being one of the most reported in the literature [1–4]. Chalcones (= 1,3-diphenylprop-2-en-1-ones), which are intermediate precursors to all flavonoid compounds, have been widely reported for their antitumor activity against a variety of tumor cell lines [5][6]. Among flavonoids, prenylated derivatives have been attracting the attention of the scientific community because of their myriad biological activities [7–9]. In fact, it has been demonstrated that isoprenylation of flavonoids significantly increased their growth inhibitory effect on human tumor cell lines [7–13]. Regarding *O*-substitutions, an aliphatic chain in the *ortho*-position of the phenyl residue adjacent to the C=O group seems to contribute significantly to increase this activity [12]. Natural prenylated chalcones, such as xanthohumol, although being structurally simple, exhibit numerous pharmacological properties [14]. Indeed, xanthohumol has been considered as a cancer

chemopreventive agent with an exceptionally broad spectrum of inhibitory mechanisms which influence carcinogenesis [15]. In the extensive range of biological activities that prenylchalcones may exert, inhibition of cell cycle and induction of apoptotic cell death are among the most intensively studied strategies for this family of compounds regarding cancer prevention and therapy [16–19].

Although the prenylation pattern may play an important role with respect to the biological activities [7–12], the pharmacological properties of chalcones were found to be also related to the presence, number, and position of OH and MeO groups in both the *A* and *B* rings [20]. In fact, MeO groups in ring *A* commonly occur in several natural and synthetic flavonoids, probably constituting a key element for their growth-inhibitory activities against tumor cell lines, since compounds with a higher degree of methoxylation are significantly more potent than the less methoxylated ones [21]. In addition, the presence of at least three adjacent MeO groups in the molecule appeared to be favorable for this activity [12]. Moreover, several studies have revealed an interesting correlation between cell-cycle regulation and methoxylation pattern. In fact, polymethoxyphenyl moieties are commonly found in a number of naturally occurring anticancer agents, such as colchicine and combretastatin A [20].

Considering this, we decided to synthesize prenylated chalcones with polymethoxyphenyl moieties. Here, we report the synthesis of a naturally occurring chalcone, **1**, and six prenylated analogs, **2**–**7** (*Fig. 1*). All synthesized compounds were evaluated for their ability to inhibit the *in vitro* growth of three human tumor cell lines, MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and A375-C5 (melanoma). Further studies were also carried out to clarify if the growth inhibitory effect was associated with cell-cycle arrest and/or induction of apoptosis. Chalcone **1** was previously isolated from several plants [22–24] and synthesized by other groups [25][26], but, to the best of our knowledge, no studies related to tumor cell-growth inhibition have been conducted. As far as we know, four of the six prenylchalcones, **2**, **3**, **5**, and **7**, are described here for the first time.

Results and Discussion. – *Synthesis.* One naturally occurring chalcone, **1**, and six prenyl analogs, **2**–**7**, were synthesized. The synthetic strategies applied for the synthesis of new chalcones were outlined in *Schemes 1–3*.

Chalcone **1** was synthesized in analogy to the procedures described in [25][26] by base-catalyzed aldol condensation of suitable acetophenone with 3,4,5-trimethoxybenzaldehyde to afford the desired chalcone in 90% yield. Compound **1** was then used as precursor for the synthesis of the *O*-prenylated derivatives **2** and **3**. This synthetic approach was based on the *O*-alkylation of **1** with prenyl or geranyl bromide in presence of tetrabutylammonium hydroxide (TBAOH; Bu_4NOH) at room temperature [12] (*Scheme 1*), leading to (prenyloxy)chalcone **2** (35% yield) and (geranyloxy)chalcone **3** (62% yield), respectively.

The *C*-prenylated derivatives **4**–**7** were prepared by base-catalyzed aldol condensation of different prenylacetophenones with 3,4,5-trimethoxybenzaldehyde. The treatment of 2′,4′,6′-trihydroxyacetophenone with prenyl or geranyl bromide was performed as reported in [27][28] to yield, as major products, the expected *C*-prenylated acetophenones [27–30]. These acetophenones were then regioselectively protected with $ClCH_2OMe$ according to the method described in [27][28] to give the

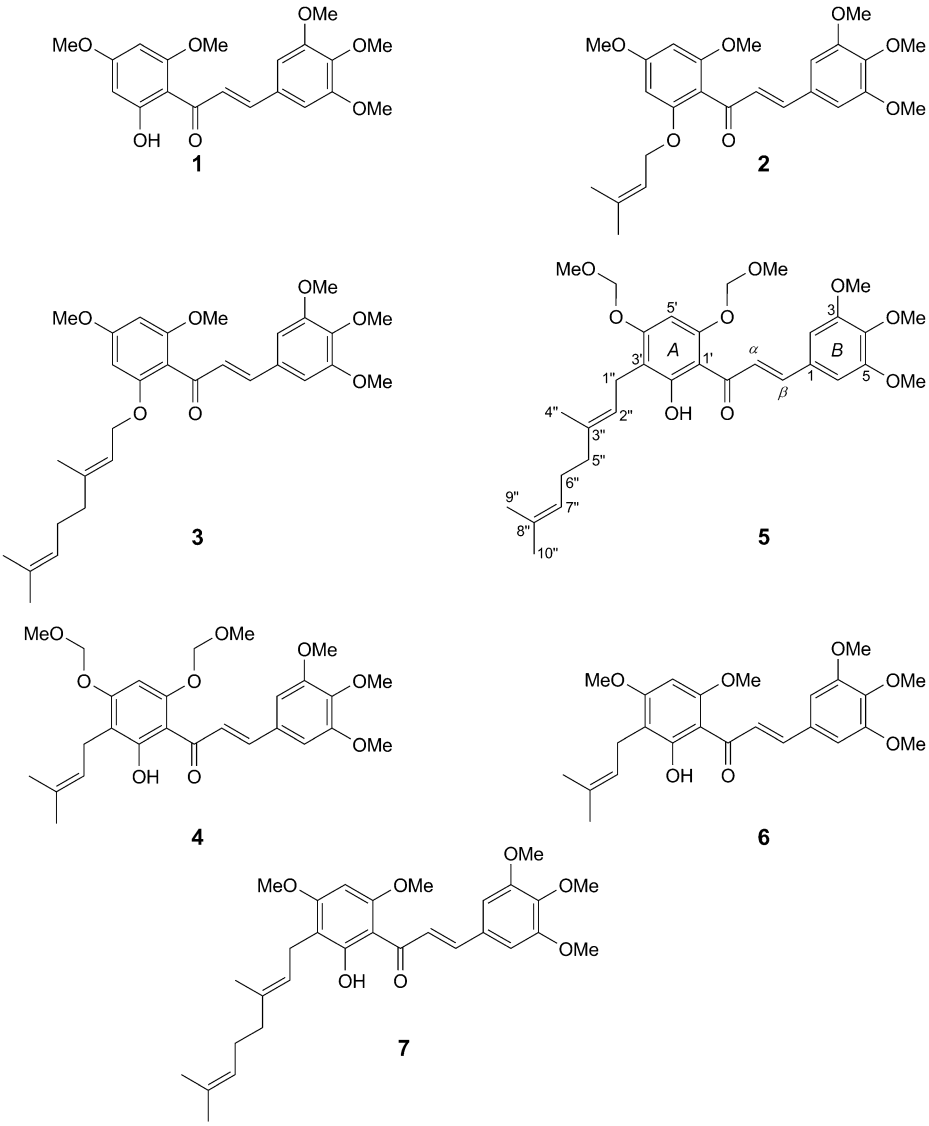
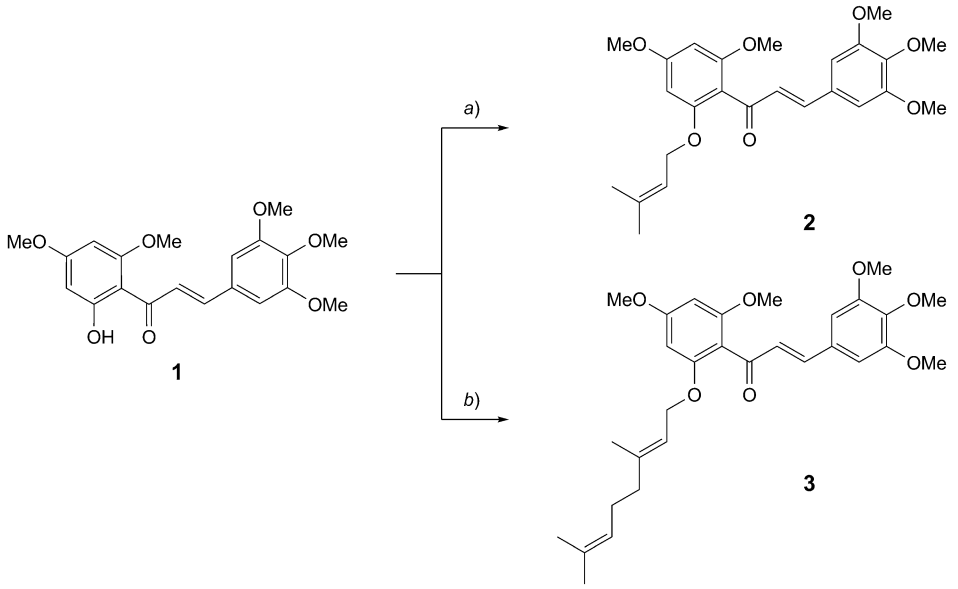


Fig. 1. *Structures of non-prenylated and prenylated chalcones* (**1** and **2**–**7**, resp.; the numbering used for NMR assignments)

predictable protected compounds [27][28][30][31] in *ca.* 30% yield. The condensation of the partially protected acetophenones with 3,4,5-trimethoxybenzaldehyde was attempted by treatment with several bases, for instance, NaOH in EtOH, and NaH in THF; however, such strategies failed (data not shown). Hence, the synthesis of **4** and **5** was achieved by aldol condensation of the corresponding protected acetophenones with 3,4,5-trimethoxybenzaldehyde, using an excess of $(^{i}Pr)_2NLi$ (LDA) [32], to afford

Scheme 1. *Synthesis of* O-*Prenylated Derivatives* **2** *and* **3**

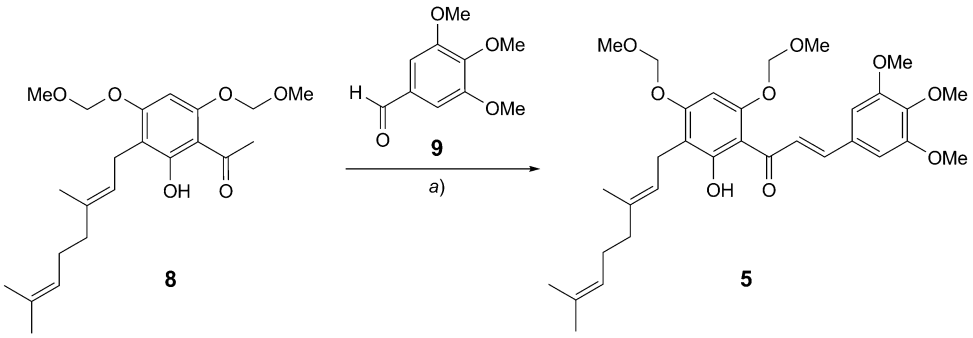


a) Prenyl bromide (=1-bromo-3-methylbut-2-ene), Bu_4NOH (TBAOH), CH_2Cl_2/toluene, r.t., 20 h. *b*) Geranyl bromide (=(*E*)-1-bromo-3,7-dimethylocta-2,7-diene), TBAOH, CH_2Cl_2/toluene, r.t., 20 h.

the prenylated chalcones **4** [33] and **5** (*Scheme 2*) in reasonable yields (46 and 47%, resp.).

Compounds **6** and **7** were also prepared by base-catalyzed aldol condensation of the appropriately substituted acetophenones with 3,4,5-trimethoxybenzaldehyde. First, 2′-hydroxy-4′,6′-dimethoxyacetophenone was prenylated by the reaction with prenyl bromide in alkaline medium in analogy to [29] to afford 2′-hydroxy-4′,6′-dimethoxy-3′-prenylacetophenone [34], or with geranyl bromide using ^{t}BuONa as a base to afford 3′-

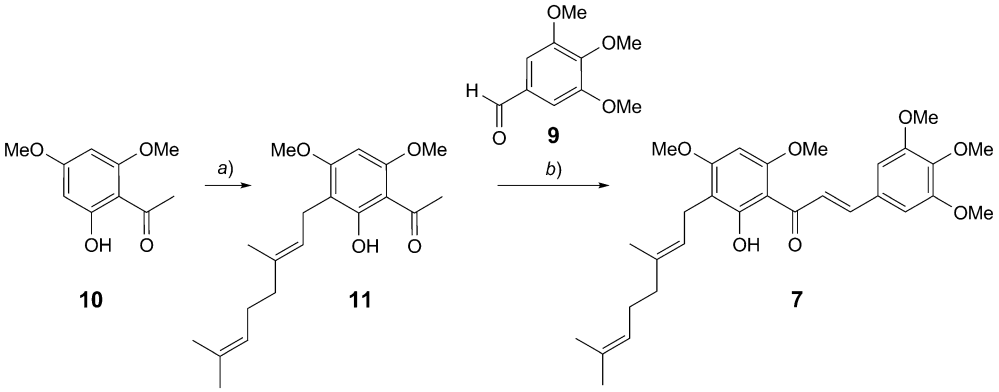
Scheme 2. *Synthesis of the* C-*Prenylated Chalcone* **5**



a) (^{i}Pr)$_2$NLi (LDA), THF, −78°, r.t., overnight.

geranyl-2′-hydroxy-4′,6′-dimethoxyacetophenone. Then, these isoprenylated acetophenones were condensed with 3,4,5-trimethoxybenzaldehyde by treatment with LDA [32] to give the prenylated chalcones **6** [34] and **7** (in 17 and 14% yield, resp., *Scheme 3*).

Scheme 3. *Synthesis of* C-*Prenylated Chalcone* **7**



a) Geranyl bromide, ^{t}BuONa, DMF, 80°, overnight. *b*) LDA, THF, −78°, r.t., overnight.

The structures of compounds **1**–**7** were established on the basis of IR, NMR, and HR-MS techniques. ^{13}C-NMR Assignments were achieved by 2D heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments. For chalcone **7**, the signal of the OH group did not appear, probably due to some exchange with DCl in the solvent used ($CDCl_3$). The coupling constants of the vinylic system ($J = 15.5–16.0$ Hz) confirmed the (*E*)-configuration of the chalcone moiety.

Biological Activity. The effects of the synthesized chalcones on the *in vitro* growth of three human tumor cell lines, MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and A375-C5 (melanoma), were evaluated according to the procedure adopted by the *National Cancer Institute* (*NCI*, USA) that uses the protein-binding dye sulforhodamine B (SRB) to assess cell growth [35][36]. By this procedure, a dose–response curve was obtained for each cell line and each test compound. The concentration that caused cell growth inhibition of 50% (GI_{50}) was determined as described in [36]. The results are compiled in *Table 1* and discussed below.

From *Table 1*, it can be seen that, among all prenylated derivatives, the 2′-(prenyloxy)chalcone **2** exhibited the lowest GI_{50} values (< 8 μM) against the three human tumor cell lines studied. Comparing the prenylated chalcones **2**, **3**, and **6** with the non-prenylated chalcone **1**, it can be concluded that the presence of a prenyl or geranyloxy group at C(2′) (compounds **2** and **3**) or a prenyloxy group at C(3′) (compound **6**) of the chalcone scaffold was associated with an increase in the growth-inhibitory effect on the MCF-7 cells, and an appearance of activity against NCI-H460 and A375-C5 cells. On the contrary, the presence of a geranyl group at C(3′) of compound **7** was associated with a complete loss of activity ($GI_{50} > 150$ μM) against the MCF-7 cell line.

Table 1. *Effect of Chalcone* **1** *and Prenylated Chalcones* **2**–**7** *on the Growth of Human Tumor Cell Lines*[a])

Compound	GI_{50}[b]) [μM]		
	MCF-7	NCI-H460	A375-C5
1	33.0 ± 4.1	> 150	> 150
2	6.2 ± 0.8	5.9 ± 0.5	7.7 ± 0.6
3	24.2 ± 1.6	22.0 ± 1.6	28.0 ± 0.8
4	8.5 ± 0.6	21.0 ± 3.1	20.3 ± 4.2
5	15.0 ± 1.8	> 150	> 150
6	19.3 ± 1.1	13.5 ± 2.6	53.0 ± 6.1
7	> 150	> 150	> 150
Doxorubicin[c])	68.8 ± 15.2	86.0 ± 9.6	86.0 ± 9.6

[a]) Data represent mean ± SEM from at least three independent experiments performed in duplicate. [b]) Concentration that was able to cause 50% of cell growth inhibition after a continuous exposure of 48 h. [c]) Doxorubicin was used as positive control, and the concentrations are presented in nM.

For chalcones **4** and **5**, the results revealed a pronounced growth-inhibitory effect against MCF-7 cells ($GI_{50} \leq 15$ μM), but a lower activity (compound **4**) or an absence of activity (compound **5**) against NCI-H460 and A375-C5 cell lines.

The comparison of the GI_{50} values of prenylated compounds **2**, **4**, and **6** with those of the corresponding geranylated derivatives **3**, **5**, and **7** indicates that the prenylation instead of geranylation is a better strategy to improve cell growth-inhibitory activity of this family of compounds.

To clarify the mechanism of action of the most active compounds ($GI_{50} < 25$ μM), flow cytometric analysis of the cell-cycle profiles and of levels of apoptosis in the MCF-7 cells treated with those compounds was carried out. Regarding the cell-cycle analysis, results showed that all tested compounds interfered with the normal cell-cycle distribution of the MCF-7 cell line. All the compounds increased the sub-G1 peak, indicating DNA degradation, characteristic for apoptosis (*Fig. 2*). Among all the compounds studied, **5** was the most potent, increasing the sub-G1 peak more than 20% with respect to the DMSO control. To confirm this observation, the most active compounds, **2**–**6**, were evaluated concerning their capability to induce apoptosis in the MCF-7 cell line. Results showed that all the compounds studied (except **4**) increased the levels of apoptosis. The geranylated derivative **5** was the most active chalcone, with the levels of apoptosis rising from 15.9% (DMSO control) to 30.6% after treatment with the GI_{50} concentration of this compound (*Table 2*).

Conclusions. – Prenylchalcone-type compounds **2**–**7** were synthesized by molecular modification of the naturally occurring chalcone **1** or *via* base-catalyzed aldol condensation of prenylated acetophenones with 3,4,5-trimethoxybenzaldehyde. These synthetic routes provided six biologically interesting prenylchalcones in reasonable yields. Greater insights into the antitumor mechanism of the action of prenylchalcones were achieved. Regarding the cell-cycle profile studies, all the tested compounds caused an increase in the levels of the sub-G1 population of cells when compared with

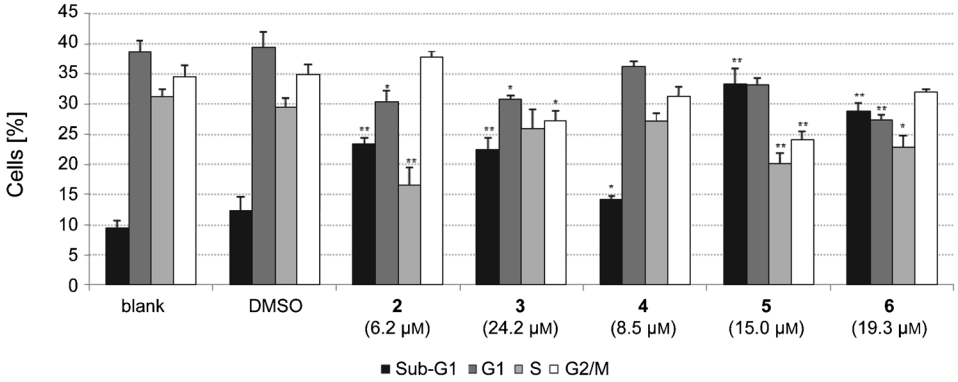


Fig. 2. *Cell-cycle analysis of MCF-7 cells treated with the chalcones with* GI_{50} *values* <25 μM. Cells were cultured with the indicated GI_{50} concentrations of the tested compounds **2**–**6** for 48 h. Appropriate controls were included: untreated cells (blank) and cells treated with the highest concentration of DMSO used to dissolve the compounds. Results represent the mean ± SEM of three independent experiments. *: $p \leq 0.05$. and **: $p \leq 0.01$, when comparing the effect of each compound with the blank (control).

Table 2. *Apoptosis Levels of MCF-7 Cells Treated with the Chalcones with* GI_{50} *values* $<25\ \mu$M

Compound[a])	Apoptotic cells[b]) (% of total cells)
Blank[c])	12.9 ± 1.29
DMSO[c])	15.9 ± 3.03
2 (6.2 μM)	20.8 ± 2.61*
3 (24.2 μM)	19.3 ± 3.14
4 (8.5 μM)	13.3 ± 3.54
5 (15.0 μM)	30.6 ± 0.49**
6 (19.3 μM)	20.8 ± 1.12**

[a]) Cells were cultured with the indicated GI_{50} concentrations of the tested compounds **2**–**6** for 48 h. [b]) Results represent the mean ± SEM of three independent experiments. *: $p \leq 0.05$ and **: $p \leq 0.01$, when comparing the effect of each compound with the blank (control). [c]) Appropriate controls were included: untreated cells (blank) and cells treated with the highest concentration of DMSO used to dissolve the compounds.

DMSO control, which was in accordance with the results obtained by the annexin-V-FITC/PI double staining assay. Using the MCF-7 cells, it was possible to conclude that the effect of these compounds on cellular growth could be at least in part caused by an apoptotic response. These results have confirmed the pro-apoptotic properties of prenylated chalcones, supporting their further study as possible chemopreventive and therapeutic agents against cancer.

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21770/2005 and SFRH/BPD/68787/2010, resp.). *K. C.* thanks for the scholarships of Rangsit University and the postgraduate program of Kasetsart University. The authors are also indebted to the *National Cancer Institute*, Bethesda, MD, USA, for the generous gift of the NCI-H460 cell line.

Experimental Part

Chemistry. General. All reagents were from commercial sources and used without purification. Column chromatography (CC): *Macherey-Nagel* silica gel *60* (SiO_2; 0.04–0.063 mm), *GraceResolv*® SiO_2 cartridges (5 g/25 ml). TLC: *Macherey-Nagel* SiO_2 *60* (GF_{254}) plates. M.p.: *Kofler* microscope; uncorrected. IR Spectra: *ATI Mattson Genesis* series FT-IR (software: WinFirst, v. 2.10) spectrophotometer; KBr microplates; $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C-NMR spectra: *Bruker Avance 300* and *500* instruments; at r.t. in $CDCl_3$; δ in ppm rel. to Me_4Si as internal standard, J in Hz. HR-EI-MS: *GC-TOF* mass spectrometer (*Waters*); in m/z; HR-ESI-MS: *APEX-Qe FT-ICR MS* mass spectrometer (*Bruker Daltonics*) or *microTOF* (focus) mass spectrometer (*Bruker Daltonics*); in m/z.

*(2*E*)-1-{2,4-Dimethoxy-6-[(3-methylbut-2-en-1-yl)oxy]phenyl}-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one* (**2**). To a soln. of chalcone **1** (0.20 g, 0.53 mmol) [25][26] and TBAOH·30 H_2O (0.87 g, 1.07 mmol) in CH_2Cl_2/toluene 10:7 (17 ml) was added prenyl bromide (93.4 µl, 0.80 mmol), and the reaction was allowed to proceed for 20 h at r.t. under gentle stirring. The reaction was quenched with H_2O and the mixture was extracted with AcOEt (3 × 50 ml). The combined org. layers were washed with H_2O, dried (Na_2SO_4), and evaporated under reduced pressure [12]. The resulting residue was purified by flash column chromatography (FC; SiO_2, hexane/AcOEt) and then by prep. TLC (SiO_2; toluene/AcOEt 8:2) to afford **2** (0.08 g, 35%). M.p. 95–99°. IR (KBr): 2996, 2927, 2840, 1643, 1602, 1580, 1500, 1456, 1415, 1380, 1336, 1314, 1271, 1244, 1221, 1200, 1154, 1121. ^1H-NMR (300 MHz, $CDCl_3$): 7.24 (*d*, J=16.0, H–C(β)); 6.86 (*d*, J=16.0, H–C(α)); 6.74 (*s*, H–C(2,6)); 6.16 (br. *s*, H–C(3′,5′)); 5.36–5.32 (*m*, H–C(2″)); 4.50 (*d*, J=6.5, CH_2(1″)); 3.87 (2*s*, MeO–C(3,5)); 3.85 (*s*, MeO–C(4)); 3.79 (*s*, MeO–C(4′)); 3.76 (*s*, MeO–C(6′)); 1.68 (*s*, H–C(5″)); 1.65 (*s*, H–C(4″)). ^{13}C-NMR (75 MHz, $CDCl_3$): 194.3 (C=O); 162.2 (C(4′)); 158.8 (C(6′)); 158.1 (C(2′)); 153.3 (C(3,5)); 144.2 (C(β)); 140.0 (C(4)); 137.5 (C(3″)); 130.6 (C(1)); 128.6 (C(α)); 119.6 (C(2″)); 111.6 (C(1′)); 105.4 (C(2,6)); 92.0 (C(3′)); 90.8 (C(5′)); 65.8 (C(1″)); 60.9 (MeO–C(4)); 56.1 (MeO–C(3,5)); 55.9 (MeO–C(6′)); 55.4 (MeO–C(4′)); 25.7 (C(5″)); 18.2 (C(4″)). HR-ESI-MS: 443.2064 ($[M+H]^+$, $C_{25}H_{31}O_7^+$; calc. 443.2070).

*(2*E*)-1-(2-{[(2*E*)-3,7-Dimethylocta-2,6-dien-1-yl]oxy}-4,6-dimethoxyphenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one* (**3**). To a soln. of **1** (0.14 g, 0.40 mmol) and TBAOH · 30 H_2O (0.66 g, 0.80 mmol) in CH_2Cl_2/toluene 1:1 (20 ml) was added geranyl bromide (115.0 µl, 0.60 mmol), and the reaction was allowed to proceed for 20 h at r.t. under gentle stirring. The reaction was quenched with H_2O, and the mixture was extracted with AcOEt (3 × 50 ml). The combined org. layers were washed with H_2O, dried (Na_2SO_4), and evaporated under reduced pressure [12]. The resulting residue was purified by FC (SiO_2, petroleum ether (PE)/AcOEt 8:2) to afford **3** (0.13 g, 62%). IR (KBr): 2926, 2843, 1645, 1599, 1500, 1456, 1418, 1381, 1333, 1268, 1244, 1152, 1120. ^1H-NMR (300 MHz, $CDCl_3$): 7.24 (*d*, J=15.6, H–C(β)); 6.87 (*d*, J=15.6, H–C(α)); 6.74 (*s*, H–C(2,6)); 6.16 (br. *s*, H–C(3′,5′)); 5.36–5.32 (*m*, H–C(2″)); 5.04–4.99 (*m*, H–C(7″)); 4.53 (*d*, J=6.2, CH_2(1″)); 3.87 (*s*, MeO–C(3,5)); 3.85 (*s*, MeO–C(4)); 3.79 (*s*, MeO–C(4′)); 3.77 (*s*, MeO–C(6′)); 2.05–1.95 (*m*, CH_2(5″,6″)); 1.65 (*s*, Me(4″)); 1.63 (*s*, Me(9″)); 1.55 (*s*, Me(10″)). ^{13}C-NMR (75 MHz, $CDCl_3$): 194.3 (C=O); 162.1 (C(4′)); 158.7 (C(6′)); 158.0 (C(2′)); 153.3 (C(3,5)); 144.2 (C(β)); 140.6 (C(3″)); 139.9 (C(4)); 131.7 (C(8″)); 130.5 (C(1)); 128.6 (C(α)); 123.6 (C(7″)); 119.5 (C(2″)); 112.0 (C(1′)); 105.4 (C(2,6)); 92.0 (C(3′)); 90.8 (C(5′)); 65.8 (C(1″)); 60.9 (MeO–C(4)); 56.1 (MeO–C(3,5)); 55.9 (MeO–C(6′)); 55.4 (MeO–C(4′)); 39.4 (C(5″)); 26.2 (C(6″)); 25.6 (C(9″)); 17.6 (C(10″)); 16.6 (C(4″)). HR-ESI-MS: 511.2706 ($[M+H]^+$, $C_{30}H_{39}O_7^+$; calc. 511.2696).

*(2*E*)-1-{3-[(2*E*)-3,7-Dimethylocta-2,6-dien-1-yl]-2-hydroxy-4,6-bis(methoxymethoxy)phenyl}-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one* (**5**). To a soln. of LDA (3 mmol) in dry THF (14 ml) was added a soln. of acetophenone **8** (1 mmol) in THF (3 ml) at −78°. The mixture was stirred at −78° for 1 h, and *3,4,5-trimethoxybenzaldehyde* (**9**; 3 mmol) in THF (3 ml) was added at −78°. The resulting mixture was stirred at −78° for 1 h, and then allowed to reach r.t. overnight. Sat. NH_4Cl soln. (30 ml) was added

dropwise, and the aq. layer was extracted with AcOEt (3 × 50 ml). The combined org. layers were washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure [32]. The resulting residue was purified by CC (SiO_2 cartridges; hexane/AcOEt) to afford **5** (0.14 g, 47%). IR (KBr): 3600–3300, 2959, 2923, 2841, 1618, 1578, 1499, 1451, 1412, 1313, 1273, 1124. ^{1}H-NMR (300 MHz, $CDCl_3$): 13.81 (*s*, HO–C(2′)); 7.87 (*d*, *J* = 15.5, H–C(*α*)); 7.72 (*d*, *J* = 15.5, H–C(*β*)); 6.87 (*s*, H–C(2,6)); 6.38 (*s*, H–C(5′)); 5.29 (*s*, CH_2O–C(6′)); 5.26 (*s*, CH_2O–C(4′)); 5.26–5.22 (*m*, H–C(2″)); 5.14–5.07 (*m*, H–C(7″)); 3.96 (*s*, MeO–C(4)); 3.92 (*s*, MeO–C(3,5)); 3.54 (*s*, *Me*OCH$_2$O–C(6′)); 3.50 (*s*, *Me*OCH$_2$O–C(4′)); 3.36 (*d*, *J* = 7.0, CH_2(1″)); 2.12–2.03 (*m*, CH_2(6″)); 2.00–1.95 (*m*, CH_2(5″)); 1.81 (*s*, Me(4″)); 1.67 (*s*, Me(9″)); 1.60 (*s*, Me(10″)). ^{13}C-NMR (75 MHz, $CDCl_3$): 193.0 (C=O); 163.8 (C(2′)); 160.8 (C(4′)); 158.1 (C(6′)); 153.4 (C(3,5)); 142.2 (C(*β*)); 140.0 (C(4)); 135.0 (C(3″)); 131.2 (C(8″)); 131.1 (C(1)); 127.1 (C(*α*)); 124.4 (C(7″)); 122.3 (C(2″)); 112.2 (C(3′)); 107.7 (C(1′)); 105.5 (C(2,6)); 95.7 (CH_2O–C(6′)); 93.9 (CH_2O–C(4′)); 92.3 (C(5′)); 61.0 (MeO–C(4)); 57.0 (*Me*OCH$_2$O–C(6′)); 56.3 (*Me*OCH$_2$O–C(4′)); 56.0 (MeO–C(3,5)); 39.8 (C(5″)); 26.7 (C(6″)); 25.7 (C(9″)); 17.6 (C(10″)); 16.1 (C(4″)). HR-ESI-MS: 571.2889 ([*M* + H]$^+$, $C_{32}H_{43}O_9^+$; calc. 571.2907).

*1-{3-[(2*E*)-3,7-Dimethylocta-2,6-dien-1-yl]-2-hydroxy-4,6-dimethoxyphenyl}ethanone* (**11**). A mixture of acetophenone **10** (0.34 g, 1.53 mmol), geranyl bromide (0.6 ml, 3.06 mmol), and *t*BuONa (0.31 g, 3.06 mmol) in dry DMF (10 ml) was gently stirred at 80° overnight. The mixture was acidified to pH of *ca.* 2 (10% HCl), and diluted with H_2O (30 ml), and the aq. layer was extracted with AcOEt (3 × 50 ml). The combined org. layers were washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure. The resulting residue was purified by FC (SiO_2; PE/Et_2O 6:4) to afford **11** (0.24 g, 47%). IR (KBr): 3600–3200, 2953, 2910, 2838, 1605, 1580, 1444, 1407, 1352, 1258, 1204, 1141, 1100. ^{1}H-NMR (300 MHz, $CDCl_3$): 14.05 (*s*, HO–C(2′)); 5.95 (*s*, CH_2(5′)); 5.29–5.19 (*m*, H–C(2″)); 5.17–5.08 (*m*, H–C(7″)); 3.89 (*s*, MeO–C(6′)); 3.85 (*s*, MeO–C(4′)); 3.26 (*d*, *J* = 7.1, CH_2(1″)); 2.61 (*s*, Me(1)); 2.11–1.91 (*m*, CH_2(5″,6″)); 1.76 (*s*, Me(4″)); 1.68 (*s*, Me(9″)); 1.60 (*s*, Me(10″)). ^{13}C-NMR (75 MHz, $CDCl_3$): 203.1 (C=O); 163.6 (C(2′)); 163.2 (C(4′)); 162.8 (C(6′)); 134.9 (C(3″)); 131.2 (C(8″)); 124.5 (C(7″)); 122.4 (C(2″)); 109.8 (C(3′)); 105.9 (C(1′)); 85.7 (C(5′)); 55.4 (MeO–C(4′,6′)); 39.7 (C(5″)); 33.1 (C(1)); 26.7 (C(6″)); 25.6 (C(9″)); 21.2 (C(1″)); 17.6 (C(10″)); 16.0 (C(4″)). HR-ESI-MS: 333.2059 ([*M* + H]$^+$, $C_{20}H_{29}O_4^+$; calc. 333.2066).

*(2*E*)-1-{3-[(2*E*)-3,7-Dimethylocta-2,6-dien-1-yl]-2-hydroxy-4,6-dimethoxyphenyl}-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one* (**7**). To a soln. of LDA (1.0 ml, 1.81 mmol) in dry THF (10 ml) was added a soln. of **11** (0.21 g, 0.60 mmol) in THF (3 ml) at −78°. The mixture was stirred at −78° for 1 h, and **9** (0.36 g, 1.81 mmol) in THF (3 ml) was added at −78°. The resulting mixture was stirred at −78° for 1 h and then allowed to reach r.t. overnight. Sat. NH_4Cl soln. (30 ml) was added dropwise, and the aq. layer was extracted with AcOEt (3 × 50 ml). The combined org. layers were washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure [32]. The resulting residue was purified by CC (SiO_2 cartridges; PE/AcOEt 7:3) to afford **7** (0.04 g, 14%). M.p. 183–186°. IR (KBr): 3500–3200, 2953, 2923, 2841, 1624, 1573, 1498, 1455, 1406, 1376, 1316, 1269, 1237, 1216, 1113. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.80 (*d*, *J* = 15.5, H–C(*α*)); 7.70 (*d*, *J* = 15.5, H–C(*β*)); 6.86 (*s*, H–C(2,6)); 6.03 (*s*, H–C(5′)); 5.22 (*t*, *J* = 7.0, H–C(2″)); 5.12–5.07 (*m*, H–C(7″)); 3.96 (*s*, MeO–C(6′)); 3.93 (*s*, MeO–C(3,4′,5)); 3.92 (*s*, MeO–C(4)); 3.33 (*d*, *J* = 7.0, CH_2(1″)); 2.09–2.04 (*m*, CH_2(6″)); 2.00–1.98 (*m*, CH_2(5″)); 1.80 (*s*, Me(4″)); 1.67 (*s*, Me(9″)); 1.60 (*s*, Me(10″)). ^{13}C-NMR (125 MHz, $CDCl_3$): 192.7 (C=O); 164.1 (C(2′)); 163.4 (C(4′)); 161.2 (C(6′)); 153.4 (C(3,5)); 141.9 (C(*β*)); 139.9 (C(4)); 134.9 (C(3″)); 131.2 (C(1)); 131.1 (C(8″)); 127.3 (C(*α*)); 124.5 (C(7″)); 122.4 (C(2″)); 110.3 (C(3′)); 106.5 (C(1′)); 105.4 (C(2,6)); 86.5 (C(5′)); 61.0 (MeO–C(4)); 56.1 (MeO–C(3,5)); 55.8 (MeO–C(6′)); 55.5 (MeO–C(4′)); 39.8 (C(5″)); 26.7 (C(6″)); 25.7 (C(9″)); 21.3 (C(1″)); 17.7 (C(10″)); 16.1 (C(4″)). HR-ESI-MS: 511.2704 ([*M* + H]$^+$, $C_{30}H_{39}O_7^+$; calc. 511.2696).

Biological Activity. Stock solns. of compounds **2**–**7** were prepared in DMSO and stored at −20°. Appropriately diluted solns. were freshly prepared just prior to the assays. Three human tumor cell lines were used: MCF-7 (breast adenocarcinoma, ECACC, UK), NCI-H460 (non-small cell lung cancer, a kind gift from NCI, Bethesda, USA), and A375-C5 (melanoma, ECACC, UK). The human tumor cell lines were grown as monolayer and routinely maintained in *RPMI-1640* medium supplemented with 5% heat-inactivated fetal bovine serum (FBS) at 37° in a humidified atmosphere containing 5% CO_2.

Growth-Inhibition Assay. The effect of chalcones on the *in vitro* growth of three human tumor cell lines, MCF-7, NCI-H460, and A375-C5 (melanoma), was evaluated according to the procedure adopted by the *National Cancer Institute* (*NCI*, USA) that uses the protein-binding dye sulforhodamine B (SRB) to assess cell growth [35][36]. Cells were plated in 96-well plates at appropriate densities to ensure exponential growth throughout the experimental period (5.0×10^3 cells/well for MCF-7 and NCI-H460, and 7.5×10^3 cells/well for A375-C5) and then allowed to adhere overnight. Attached cells were exposed for 48 h to five serial dilutions of each compound (150, 75, 37.5, 18.75, and 9.37 μM for compounds **1**, **6**, and **7**, or 150, 50, 16.67, 5.56, and 1.85 μM for compounds **2**–**5**). After this incubation period, adherent cells were fixed *in situ* with Cl_3CCOOH (TCA), washed, and stained with SRB [36][37]. The bound stain was solubilized, and the absorbance was measured at 492 nm with a plate reader (*Biotek Instruments Inc. PowerWave XS*, Winooski, USA). A dose–response curve was obtained for each cell line with each test compound, and the concentration that caused cell-growth inhibition of 50% (GI_{50}, *i.e.* concentration of compound that inhibited 50% of the net cell growth), was determined as described in [36]. The effect of the vehicle solvent (DMSO) on the growth of these cell lines was evaluated in preliminary experiments by exposing untreated control cells to the maximum concentration of DMSO used in each assay. Final concentrations of DMSO did not interfere with the biological activity tested (data not shown).

Cell-Cycle Analysis. MCF-7 Cells were plated in 6-well plates (5.0×10^4 cells/ml) and incubated at 37° for 24 h. Exponentially growing cells were then incubated with the compounds **2**–**6** at their respective GI_{50} concentrations (previously determined with the SRB assay; *cf.* data in *Table 1*). Untreated cells (control) or cells treated with the compound's solvent (DMSO) were included. DMSO was used at the highest concentration used in the experiments. Following 48-h treatment, cells were centrifuged and fixed in 70% EtOH at 4° for at least 12 h and subsequently resuspended in PBS containing 0.1 mg/ml RNase A and 5 μg/ml propidium iodide. Cellular DNA content, for cell cycle distribution analysis, was measured by flow cytometry with an *Epics XL-MCL Coulter* flow cytometer (Brea, CA, USA) plotting at least 20,000 events per sample, as described in [38]. The percentage of cells in the sub-G1, G1, S, and G2/M phases of the cell cycle was determined by using the *FlowJo 7.2* software (*Tree Star, Inc.*, Ashland, OR, USA) after cell-debris exclusion.

Analysis of Cellular Apoptosis. MCF-7 Cells were incubated with the compounds as described previously for cell cycle-analysis assay. Cells were then harvested and the *Human Annexin-V-FITC/PI* apoptosis kit (*Bender MedSystems*, A-Vienna) was used according to the manufacturer's instructions to detect apoptotic cells. Flow cytometry was carried out with an *Epics XL-MCL Coulter* flow cytometer (Brea, CA, USA) plotting at least 20,000 events per sample. Data obtained from the flow cytometer was analyzed by using the *FlowJo 7.2* software (*Tree Star, Inc.*, Ashland, OR, USA) as described in [39][40].

Statistical Analysis. All exper. data are presented as means ± SEM from at least three independent experiments (most of them performed in duplicate). Statistical analyses was carried out using an unpaired *Student*'s *t*-test. All analyses were performed comparing cells treated with compounds with blank cells (cells treated with medium only).

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