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Solid-phase synthesis of 2'-hydroxychalcones. Effects on cell growth inhibition, cell cycle and apoptosis of human tumor cell lines

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ABSTRACT

Thirty-one 2'-hydroxychalcones were prepared via solid-phase synthesis by base-catalyzed aldol condensation of substituted 2'-hydroxyacetophenones and benzaldehydes. Chalcones were tested for their growth inhibitory activity in three human tumor cell lines (MCF-7, NCI-H460 and A375-C5) using the SRB assay. Results revealed that several of the tested compounds caused a pronounced dose-dependent growth inhibitory effect on the tumor cell lines studied in the low micromolar range. To gain further insight on the cellular mechanism of action of this class of compounds, studies of their effect on cell cycle profile as well as on induction of cellular apoptosis were also carried out. Generally, the tested chalcones interfered with the cell cycle profile and increased the percentage of apoptotic MCF-7 cells. The results here presented may help to identify new chalcone-like structures with optimized cell growth inhibitory activity which may be further tested as potential antitumor agents.

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1. Introduction

Flavonoids represent an outstanding class of naturally occurring compounds, which reveal to be an important source of interesting biological activities.^{1–3} Among this family of compounds, chalcones represent one of the major classes of natural products and have long been recognised for their myriad of biological activities,^{1–6} namely antitumor. Interestingly, the antitumor properties of chalcones were found to be related to the presence, the number and the position of hydroxyl, methoxyl and halogen substituents in both A and B rings.⁷ The occurrence of at least three adjacent methoxyl groups appeared to be favourable for their growth inhibitory effect in tumor cell lines.⁸ Moreover, several studies have demonstrated an interesting association between interference in cell cycle and the methoxylation pattern.⁵ Actually, a polymethoxyphenyl moiety is commonly found in a number of naturally occurring anticancer agents, such as colchicine and combretastatin A.⁵ 2',4'-

Dihydroxychalcone is a naturally occurring compound which is well known for its in vitro antitumor activities, namely by inducing cytotoxicity in gastric cancer cells (MGC-803) in a dose- and timedependent manner, causing MGC-803 cell shrinkage and apoptotic body formation, typical characteristics of apoptosis and cell cycle arrest in the G2/M phase.^{9,10} Furthermore, 2',4'-dihydroxychalcone significantly increased caspase-3 activity and decreased survivin mRNA expression suggesting that it induces apoptosis in gastric cancer cells via down-regulation of survivin mRNA expression.^{9,10} According to these results the substitution profile 2',4'-dihydroxy in ring A of the chalcone scaffold seems to be interesting to obtain chalcones with growth inhibitory activity in tumor cell lines.

Considering this, we planned to synthesize a series of structural related chalcones possessing both halogen and methoxyl groups on B ring and hydroxyl and alkyl groups on A ring to further investigate 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). To get some insights into the cellular mechanism of action of these chalcones, the most active derivatives were also studied regarding their capacity to cause cell cycle arrest and to induce apoptosis.

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2. Results and discussion

2.1. Chemistry

This article focuses on the synthesis of specific methoxylated and halogenated chalcones. A series of thirty-one chalcones and two flavanones was prepared in solid-phase via base-catalyzed aldol reaction using three 2'-hydroxyacetophenones and twelve benzaldehydes as building blocks. The synthesis was performed according to the general reaction pathway outlined in Scheme 1. The diversity of the present small library was planned based on the scaffold of some bioactive chalcones,^{5,7,9,10} and on the commercial availability of the building blocks. To our best knowledge twenty-three (**1**, **12–33**) of the thirty-three synthesized compounds are being described for the first time.

Before carrying out the synthesis of the 2'-hydroxychalcones some tests were done in order to optimize the reaction conditions. Once the best conditions had been set up, we went on performing the solid-phase synthesis using a Büchi Syncore synthesizer. Thus, the immobilization of 2'-hydroxyacetophenones on the solid support was carried out using diisopropylethylamine (DIEA) in CH₂Cl₂, and the condensation of the solid-supported acetophenones with benzaldehydes was achieved using NaOH in THF/MeOH. The use of 2-chlorotrityl chloride resin for the immobilization of 2'hydroxyacetophenones was related to the high loading and mild acidic cleavage conditions presented for this type of resins over other polymeric supports.¹¹

The solid-phase synthesis of 2'-hydroxychalcones began with the treatment of the 2-chlorotrityl chloride resin with 2'-hydroxyacetophenones under basic conditions, in order to provide the solid-supported acetophenones. The resin-loading was 70-90%, as determined by mass difference of vacuum dried resins. To confirm the immobilization of acetophenones on the solid support, KBr pellets of the solid-supported acetophenones were analysed by IR. The appearance of a characteristic band of carbonyl stretch for acetophenones at 1631–1625 cm⁻¹ revealed their immobilization on the resin. The treatment of this solid-supported acetophenones with substituted benzaldehydes under basic conditions afforded the solid-supported chalcones. The exposure of these immobilized chalcones to 2% TFA in CH₂Cl₂ promoted the cleavage of 2-chlorotrityl resin to afford a series of thirty-one chalcones and two flavanones in moderate yields (Scheme 1).¹² The cleavage product was purified and the yield after two steps (loading and cleavage) was determined based on an estimated resin functionalization of 1.0 mmol Cl/g. The flavanones **32** and **33** were obtained by cyclization of the correspondent 2'-hydroxychalcones, induced by the acid conditions used during the cleavage. This kind of events has already been described for similar conditions.¹³ In addition, 2',4,4'-trihydroxy-2-methoxymethoxychalcone (**1**) was obtained instead of 2',4'-dihydroxy-2,4-bis(methoxymethoxy)chalcone revealing that deprotection of position 4 occurred during the acidic cleavage.

The structure elucidation of 2'-hydroxychalcones and flavanones (purity $\ge 95\%$) was established by IR, HRMS and NMR techniques. On the other hand, compounds with lower purity were only characterized by HRMS and ¹H NMR. The spectroscopic data of compounds **3** and **8–10** were in agreement with those found in literature.^{14,15} The coupling constants for the protons of the vinylic system ($J_{H\alpha-H\beta} = 15.3-16.1$ Hz) confirmed the (*E*)-configuration. In fact, this synthetic route of chalcones is quite attractive since it generates the (*E*)-isomer in a diastereoselective way, usually in fairly good yields, from appropriate substituted 2'-hydroxyacetophenones and benzaldehydes.

2.2. Biological activity

The effect of chalcones on the in vitro growth of three human tumor cell lines, MCF-7 (breast adenocarcinoma), NCI-H460 (nonsmall cell lung cancer) and A375-C5 (melanoma) was evaluated according to the procedure adopted by the National Cancer Institute (NCI, USA) which uses the protein-binding dye sulforhodamine B (SRB) to assess cell growth.^{16,17} Based on this procedure, a dose–response curve was obtained for each cell line with each test compound and the concentration that caused cell growth inhibition of 50% (GI₅₀, corresponding to the concentration of compound that inhibited 50% of net cell growth), was determined as described elsewhere.¹⁷

To clarify the mechanism of action of the most active compounds (GI₅₀ <10.5 μ M), the MCF-7 cell line was used for treatment with the compounds, and cells were further processed in order to analyze alterations in the cell cycle profile and in the levels of apoptosis.

The synthesized chalcones and flavanones with purity values greater than 95% were evaluated for their capacity to inhibit the in vitro growth of three human tumor cell lines, MCF-7, NCI-H460 and A375-C5. Results showed in Table 1 indicate that from the studied chalcones the most potent were the 2',4'-dihydroxy-3,4,5-trimethoxychalcone (**2**), 4-benzyloxy-2',4'-dihydrochalcone



Scheme 1. Synthetic strategy for solid-phase synthesis of 2'-hydroxychalcones. Reagents and conditions: (a) DIEA, CH₂Cl₂/DMF, rt, 48 h; (b) NaOH, THF/MeOH, rt, 48 h; (c) 2% TFA/CH₂Cl₂, rt, 30 min (the numbering used concerns the NMR assignments).

able 1	
ffect of 2'-hydroxychalcones (C1-C31) and flavanones (F1-F2) on the growth of three human tumor cell lines	



Compound	R ₁	R ₂	R ₃	R ₄	R ₅	GI ₅₀ (µM)		
						MCF- 7	NCI-H460	A375- C5
1	Н	OCH ₂ OCH ₃	Н	Н	Н	ND	ND	ND
2	Н	Н	OCH ₃	OCH ₃	OCH ₃	7.8 ± 0.5	8.2 ± 0.3	8.1 ± 1.6
3	Н	OCH ₃	Н	OCH ₃	Н	19.5 ± 1.0	37.0 ± 4.0	30.3 ± 3.9
4	Н	OCH ₃	Н	OCH ₃	OCH ₃	31.8 ± 2.4	60.0 ± 2.5	34.8 ± 7.1
5	Н	Н	Н	Br	Н	ND	ND	ND
6	Н	Н	Н	CF ₃	Н	ND	ND	ND
7	Н	Н	Н	OBn	Н	8.0 ± 0.5	10.5 ± 1.3	9.0 ± 1.1
8	Н	Н	Н	Cl	Н	ND	ND	ND
9	Н	Cl	Н	Cl	Н	ND	ND	ND
10	Н	Н	Н	OCH ₃	Н	ND	ND	ND
11	Н	Н	Н	F	Н	7.3 ± 0.6	15.3 ± 2.2	14.1 ± 2.1
12	CH ₂ CH ₂ CH ₃	OCH ₂ OCH ₃	Н	OCH ₂ OCH ₃	Н	18.2 ± 2.1	24.3 ± 2.2	35.0 ± 6.4
13	CH ₂ CH ₂ CH ₃	Н	OCH ₃	OCH ₃	OCH_3	4.1 ± 0.5	8.3 ± 0.9	8.2 ± 1.6
14	CH ₂ CH ₂ CH ₃	OCH ₃	Н	OCH ₃	Н	ND	ND	ND
15	CH ₂ CH ₂ CH ₃	OCH ₃	Н	OCH ₃	OCH ₃	9.1 ± 0.6	24.5 ± 3.7	23.0 ± 1.9
16	CH ₂ CH ₂ CH ₃	Н	Н	Br	Н	ND	ND	ND
17	CH ₂ CH ₂ CH ₃	Н	Н	CF ₃	Н	ND	ND	ND
18	CH ₂ CH ₂ CH ₃	Н	Н	Cl	Н	ND	ND	ND
19	CH ₂ CH ₂ CH ₃	Н	Н	OCH ₃	Н	13.3 ± 1.9	24.7 ± 1.9	46.3 ± 9.4
20	CH ₂ CH ₂ CH ₃	Н	Н	F	Н	ND	ND	ND
21	CH ₂ CH ₂ CH ₃	Cl	Н	Н	Н	7.1 ± 0.6	12.5 ± 1.2	14.2 ± 1.7
22	CH ₃	OCH ₂ OCH ₃	Н	OCH ₂ OCH ₃	Н	32.7 ± 5.4	>150	>150
23	CH ₃	Н	OCH ₃	OCH ₃	OCH_3	ND	ND	ND
24	CH ₃	OCH ₃	Н	OCH ₃	OCH ₃	ND	ND	ND
25	CH ₃	Н	Н	Br	Н	ND	ND	ND
26	CH ₃	Н	Н	CF ₃	Н	19.3 ± 1.7	20.3 ± 4.6	24.7 ± 4.7
27	CH ₃	Н	Н	OBn	Н	ND	ND	ND
28	CH ₃	Н	Н	Cl	Н	34.5 ± 2.7	43.0 ± 4.1	41.6 ± 1.4
29	CH ₃	Cl	Н	Cl	Н	10.5 ± 2.7	22.5 ± 1.3	21.4 ± 1.8
30	CH ₃	Н	Н	OCH ₃	Н	ND	ND	ND
31	CH ₃	Н	Н	F	Н	ND	ND	ND
32	CH ₂ CH ₂ CH ₃	OCH ₃	Н	OCH ₃	OCH ₃	18.8 ± 1.1	47.0 ± 10.1	32.0 ± 3.0
33	CH ₃	OCH ₃	Н	OCH ₃	Н	12.5 ± 1.2	>150	>150

Results are given in concentrations that were able to cause 50% of cell growth inhibition (GI_{50}) after continuous treatment for 48 h and represent mean <u>+</u> SEM of at least three independent experiments performed in duplicated. Doxorubicin was used as positive control: GI_{50} (MCF-7) = 68.8 ± 15.2 nM; GI_{50} (NCI-H460) = 86.0 ± 9.6 nM; GI_{50} (A375-C5) = 91.5 ± 8.0 nM. ND = not determined.

(7), and 2',4'-dihydroxy-3,4,5-trimethoxy-3'-propylchalcone (13) which presented GI_{50} values ranging from 4.1 to 10.5 μ M in the three human tumor cell lines studied.

Considering the substitution patterns of ring B, some interesting results were observed. Regarding the methoxylated chalcones, when comparing GI_{50} values of 3,4,5-trimethoxychalcones 2 and 13 with the corresponding 2,4,5-trimethoxychalcones 4 and 15, the results revealed that the presence of three methoxyl groups on positions 3,4,5 is more favourable for their growth inhibitory activity than the presence on positions 2,4,5. These results suggest the importance of a trimethoxyphenyl moiety in positions 3,4,5 of the chalcone scaffold for this activity. Moreover, the comparison of the GI_{50} values of chalcone 15 (GI_{50} = 9.1–24.5 $\mu M)$ with the corresponding flavanone **32** ($GI_{50} = 18.8-47.0 \mu M$) suggest the importance of the chalcone scaffold for the cell growth inhibitory effect. Regarding the growth inhibitory activity for the halogenated chalcones, 26, 28 and 29, the results showed that the presence of 4trifluoromethyl substituent (26) was more favourable than a 4chloride substituent (28) for the studied effect. Furthermore, comparing compounds 28 and 29, the presence of additional chlorine at C-2 (**29**) was associated with an increase of the growth inhibitory effect in the three human tumor cell lines.

Considering the substitution patterns of ring A, the comparison of GI₅₀ values of chalcones **4** and **15** revealed that the presence of a 3'-propyl side chain was responsible for a substantial improvement in the growth inhibitory activity in the three human tumor cell lines studied. Additionally, the presence of a propyl side chain (chalcone **12**) instead of a 3'-methyl group (chalcone **22**) was also associated with an increase in the growth inhibitory effect of MCF-7 cells and an appearance of activity in NCI-H460 and A375-C5 cell lines. These results suggest that the strategy of alkylation may play an interesting role in the cell growth inhibitory activity caused by this class of compounds in human tumor cell lines.

By comparing the GI_{50} values obtained for each compound in the three human tumor cell lines, we observed that the MCF-7 cell line was the most sensitive to the compounds studied, in particular to compounds **22** and **33**.

The most active compounds ($GI_{50} < 10.5 \ \mu$ M) were selected to study their effect on cell cycle profile and apoptosis. Therefore, cells were treated for 48 h with compounds **2**, **7**, **11**, **13**, **15**, **21**

and **29** at their respective GI₅₀ concentrations and then harvested and further processed for cell cycle analysis and apoptosis detection by flow cytometry. Results showed that the majority of the tested chalcones interfered with the cell cycle distribution of MCF-7 cells. In addition, compounds 2, 7, 11 increased the sub-G1 peak, which is indicative of DNA degradation, characteristic of apoptosis (Fig. 1). Among the studied compounds, the 4-fluoro-2',4'-dihydroxychalcone (11) was the most potent, increasing the sub-G1 peak more than 11% in relation to DMSO control. In order to confirm this observation, the most active compounds (2, 7, 11, 13, 15, 21 and 29) were further evaluated concerning their capability to induce apoptosis in the MCF-7 cell line. Results from the Annexin-V-FITC/PI assay showed that compounds 2 and 11 increased the levels of apoptosis. Once more, the 4-fluoro-2',4'-dihydroxychalcone (11) was the most active compound, causing a significant increase in the levels of apoptosis rising from 11.1% (DMSO control) to 23.9% (Table 2).

3. Conclusions

A series of thirty-one substituted 2'-hydroxychalcones was synthesized through a solid-phase approach. Most of the studied compounds inhibited the growth of the three human tumor cell lines, being **2**, **7**, **11**, **13** and **21** the most active chalcones. In addition, the growth inhibitory effect of chalcone **11** could be associated, at least in part, to an induction of cellular apoptosis and/or to alterations in the cell cycle profile of MCF-7 cells. In conclusion, the present study allows the identification of compounds with antitumor activity and brought some structure–activity data that will aid in the design of more active chalcones.

4. Experimental

4.1. Chemistry

The phenol groups of 2,4-dihydroxybenzaldeyde were protected with chloromethylmethyl ether (MOMCl) according to the procedure described elsewhere,¹⁸ to afford 2,4-bis(methoxymethoxy)benzaldehyde in 59% yield. Solid-phase synthesis of chalcones was performed using a Büchi Syncore synthesizer. The reaction vessels were oven-dried and the resin (2-chlorotritylchlo-

Table 2

Apoptosis levels of MCF-7 cells treated with chalcones with $GI_{50} \leq 10.5 \ \mu M$

Compounds	Apoptosis (% of total cells)
Blank	12.9 ± 1.29
DMSO	11.1
2 (7.8 μM)	20.0 ± 2.63
7 (8.0 μM)	15.4 ± 1.32
11 (7.3 μM)	$23.9 \pm 2.73^{*}$
13 (4.1 μM)	14.0 ± 1. 97
15 (9.1 μM)	13.3 ± 2.01
21 (7.1 μM)	14.8 ± 1.65
29 (10.5 μM)	16.6 ± 2.54

Appropriate controls were included: untreated cells (blank) and cells treated with the highest concentration of DMSO used to dissolve the compounds (DMSO). Results represent the mean ± SEM of three independent experiments, except for DMSO control which results from only two independent experiments.

 * Represents $p \leqslant$ 0.05 when comparing the effect of each compound with the blank (control).

ride), the 2'-hydroxyacetophenones and benzaldehydes were dried over P₂O₅ in a desiccator under vacuum before use. IR was used to monitor and optimize the reactions performed in solid-phase (Bomem M102 spectrometer, software: ACD/Labs 6.00). Purifications of 2'-hydroxychalcones were carried out by flash chromatography using GraceResolv[®] silica gel cartridges (5 g/25 mL). Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were measured on an ATI Mattson Genesis series FTIR (software: WinFirst, v. 2.10) spectrophotometer in KBr microplates (cm⁻¹). ¹H and ¹³C NMR spectra were taken in CDCl₃ at room temperature, on Bruker Avance 300 and 500 instruments (300.13 or 500.13 MHz for ¹H and 75.47 or 125.77 MHz for ¹³C). Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) used as an internal reference; ¹³C NMR assignments were made by 2D (HSQC and HMBC) NMR experiments (long-range ¹³C-¹H coupling constants were optimized to 7 Hz). ESI-HRMS experiments were performed at C.A.C.T.I.-University of Vigo (Spain) on an APEXQe FT-ICR MS (Bruker Daltonics, USA), equipped with a 7T actively shielded magnet. Ions were generated using a Combi MALDI-electrospray ionization source. Data acquisition was performed using the ApexControl software version 3.0.0, and data processing was performed using the Data Analysis software,



Figure 1. Cell cycle analysis of MCF-7 cells treated with the most active chalcones ($GI_{50} < 10.5 \mu M$). Cells were cultured with the GI_{50} concentration of the tested compounds for 48 h. Appropriate controls were included: untreated cells (blank) and cells treated with the highest concentration of DMSO used to dissolve the compounds (DMSO). Results represent the mean ± SEM of three independent experiments, except for DMSO control and compound **7** in which case only two independent experiments were performed. * Represents $p \leq 0.05$ and ** represents $p \leq 0.01$ when comparing the effect of each compound with the blank (control).

version 4.0 both from Bruker Daltonics. Analytical HPLC analyses were performed on a Finnigan Surveyor (Thermo Electron Corporation, USA) equipped with a diode array detector TSP UV6000LP. The separation was carried out on a $250 \times 4.6 \text{ mm}$ i.d. GraceSmart RP18 (5 µm) (Grace Davison Discovery Sciences, Belgium). LC analysis was performed by isocratic elution using a mixture of MeOH:H₂O as mobile phase and the flow rate was set at 1 mL/ min. The injected volume was 20 µL and the eluent was monitored at 190-800 nm. Xcalibur 2.0 SUR 1 software (Thermo Electron Corporation 1998–2005) managed chromatographic data. Methanol (HPLC grade) was obtained from Carlo Erba Reagents, Val de Reuil, Italy and HPLC grade water (Simplicity[®] UVUltrapure Water System, Millipore Corporation, USA). Prior to use, mobile phase solvents were degassed in an ultrasonic bath for 15 min. All commercially available reagents were purchased from Sigma Aldrich Co. Reagents and solvents were purified and dried according to the usual procedures described elsewhere.¹⁹ The following materials were synthesized and purified by the described procedures.

4.1.1. General procedure for the solid-phase synthesis of 2'hydroxychalcones and flavanones (1–33)

In a 24 reaction vessels of Büchi Syncore synthesizer a mixture of 2'-hydroxyacetophenones (10 mmol), DIEA (2 mmol) and 2chlorotritylchloride resin (1.0–1.5 mmol Cl/g) in anhydrous CH₂Cl₂/DMF (3:0.5 mL) was stirred at room temperature under nitrogen atmosphere for 48 h to provide the solid-supported acetophenones. These immobilized acetophenones were washed with anhydrous THF (2 \times 3 mL), anhydrous MeOH (3 \times 3 mL) and anhydrous CH_2Cl_2 (4 × 3 mL) and dried under vacuum for 24 h. To the solid-supported acetophenones (1 mmol) were added the appropriate benzaldehydes (10 mmol), a solution of 4% NaOH in anhydrous MeOH (2 mmol) and anhydrous THF (3 mL). The reaction mixture was gentle stirred at room temperature under nitrogen atmosphere for 48 h to give the solid-supported chalcones. These immobilized chalcones were washed and dried in the same sequence as the first step described above. The solid-supported chalcones were exposed to 2% TFA in CH₂Cl₂ at room temperature under nitrogen atmosphere for 30 min. The cleaved 2-chlorotrityl resin was filtered and washed with 2% TFA:CH₂Cl₂ (2×1 mL) and CH_2Cl_2 (5 × 3 mL) and the solvent was evaporated under reduced pressure. The resulting residue was then purified by flash chromatography cartridges (SiO₂, *n*-hexane:EtOAc) to afford the 2'hydroxychalcones 1-31 and flavanones 32 and 33.

4.1.2. 2',4,4'-Trihydroxy-2-methoxymethoxychalcone (1)

Yield: 14%; ¹H NMR (CDCl₃, 300 MHz): δ 13.55 (1H, s, 2'-OH), 8.20 (1H, d, *J* = 15.6 Hz, H-β), 7.82 (1H, d, *J* = 9.4 Hz, H-6'), 7.58 (1H, d, *J* = 8.5 Hz, H-6), 7.54 (1H, d, *J* = 15.6 Hz, H-α), 6.71 (1H, d, *J* = 2.4 Hz, H-3), 6.54 (1H, dd, *J* = 8.5, 2.4 Hz, H-5), 6.44–6.41 (1H, m, H-5'), 6.42 (1H, d, *J* = 2.3 Hz, H-3') 5.28 (2H, s, $-OCH_2$), 3.52 (3H, s, $-OCH_3$); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₇H₁₇O₆ (M+H)⁺: 317.1020; found: 317.1020.

4.1.3. 2',4'-Dihydroxy-3,4,5-trimethoxychalcone (2)

Yield: 35% as yellow solid; mp: 199–200 °C; IR (KBr) ν_{max} : 3316, 2913, 2885, 2819, 1615, 1571, 1491, 1450, 1364, 1318, 1266, 1194, 1171, 1117; ¹H NMR (CDCl₃, 500 MHz): δ 13.38 (1H, s, 2'-OH), 7.85 (1H, d, *J* = 8.6 Hz, H-6'), 7.81 (1H, d, *J* = 15.3 Hz, H-β), 7.45 (1H, d, *J* = 15.3 Hz, H-α), 6.87 (2 × 1H, s, H-2,6), 6.45 (1H, dd, *J* = 8.6, 2.5 Hz, H-5'), 6.43 (1H, d, *J* = 2.5 Hz, H-3'), 3.94 (2 × 3H, s, 3,5-OCH₃), 3.91 (3H, s, 4-OCH₃); ¹³C NMR (CDCl₃, 125 MHz): δ 191.7 (C=O), 166.5 (C-2'), 162.6 (C-4'), 153.5 (C-3,5), 144.7 (C-β), 140.6 (C-4), 131.9 (C-6'), 130.2 (C-1), 119.4 (C-α), 114.5 (C-1'), 107.7 (C-5'), 105.8 (C-2,6), 103.8 (C-3'), 61.0 (4-OCH₃), 56.3 (3,5-OCH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₈H₁₉O₆ (M+H)⁺: 331.1176; found: 331.1177.

4.1.4. 2',4'-Dihydroxy-2,4-dimethoxychalcone (3)

Yield: 51% as yellow solid; mp:81–84 °C; IR (KBr) v_{max} : 3461, 2930, 2817, 1622, 1601, 1548, 1499, 1450, 1351, 1302, 1255, 1196, 1138, 1106, 1016; ¹H NMR (CDCl₃, 500 MHz): δ 13.69 (1H, s, 2'-OH), 8.11 (1H, d, *J* = 15.6 Hz, H- β), 7.81 (1H, d, *J* = 9.6 Hz, H-6'), 7.60 (1H, d, *J* = 15.6 Hz, H- α), 7.56 (1H, d, *J* = 8.8 Hz, H-6), 6.58–6.51 (2 × 1H, m, H-3,5), 6.49–6.44 (2 × 1H, m, H-3',5'), 3.90 (3H, s, 4-OCH₃), 3.86 (3H, s, 2-OCH₃); ¹³C NMR (CDCl₃, 125 MHz): δ 192.6 (C=O), 166.3 (C-2'), 163.2 (C-4), 162.8 (C-4'), 160.5 (C-2), 140.3 (C- β), 131.9 (C-6'), 131.3 (C-6), 118.2 (C- α), 117.0 (C-1),114.5 (C-1'), 110.5 (C-5), 107.6 (C-5'), 105.5 (C-3), 103.6(C-3'), 55.5 (2,4-OCH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₇H₁₇O₅ (M+H)⁺: 301.1071; found: 301.1070.

4.1.5. 2',4'-Dihydroxy-2,4,5-trimethoxychalcone (4)

Yield: 53% as orange solid; mp: 186–188 °C; IR (KBr) ν_{max} : 3395, 2936, 2912, 2823, 2715, 1641, 1602, 1571, 1509, 1457, 1398, 1364, 1332, 1290, 1237, 1199, 1118, 1021; ¹H NMR (CDCl₃, 500 MHz): *δ* 8.18 (1H, d, *J* = 15.4 Hz, H-β), 7.84 (1H, d, *J* = 8.3 Hz, H-6'), 7.53 (1H, d, *J* = 15.4 Hz, H-α), 7.12 (1H, s, H-6), 6.53 (1H, s, H-3), 6.43 (1H, dd, *J* = 8.3, 2.5 Hz, H-5'), 6.42 (1H, s, H-3'), 3.96 (3H, s, 2-0CH₃), 3.93 (3H, s, 5-0CH₃), 3.92 (3H, s, 4-0CH₃); ¹³C NMR (CDCl₃, 125 MHz): *δ* 192.4 (C=O), 166.4 (C-2'), 162.3 (C-4'), 154.9 (C-2), 152.7 (C-5), 143.4 (C-4), 140.0 (C-β), 131.8 (C-6'), 118.0 (C-α), 115.3 (C-1), 114.5 (C-1'), 111.7 (C-6), 107.4 (C-5'), 103.7 (C-3'), 104.7 (C-3), 56.6 (2-0CH₃), 56.3 (4-0CH₃), 56.1 (5-0CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₈H₁₉O₆ (M+H)⁺: 331.1176; found: 331.1175.

4.1.6. 4-Bromo-2',4'-dihydroxychalcone (5)

Yield: 20%; ¹H NMR (CDCl₃, 300 MHz): *δ* 13.28 (1H, s, 2'-OH), 7.85 (1H, d, *J* = 8.3 Hz, H-6'), 7.82 (1H, d, *J* = 15.5 Hz, H-β), 7.61– 7.46 (2 × 1H, m, H-2,6), 7.54 (1H, d, *J* = 15.5 Hz, H-α), 7.34–7.26 (2 × 1H, m, H-3,5), 6.45 (1H, dd, *J* = 8.3, 2.0 Hz, H-5'), 6.43 (1H, s, H-3'). ESI-HRMS (+) *m/z*: Anal. Calcd for $C_{15}H_{12}BrO_3$ (M+H)⁺: 318.9964; found: 318.9964.

4.1.7. 2',4'-Dihydroxy-4-trifluoromethylchalcone (6)

Yield: 9%; ¹H NMR (CDCl₃, 300 MHz): δ 13.21 (1H, s, 2'-OH), 7.88 (1H, d, *J* = 15.6 Hz, H- β), 7.84 (1H, d, *J* = 8.9 Hz, H-6'), 7.77– 7.58 (2 × 1H, m, H-2,6), 7.63 (1H, d, *J* = 15.6 Hz, H- α), 7.35 (2 × 1H, d, *J* = 8.5 Hz, H-3,5), 6.49 (1H, dd, *J* = 8.9, 2.4 Hz, H-5'), 6.45 (1H, s, H-3'); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₆H₁₂F₃O₃ (M+H)⁺: 309.0733; found: 309.0732.

4.1.8. 4-Benzyloxy-2',4'-dihydroxychalcone (7)

Yield: 58% as yellow solid; mp: 162–165 °C; IR (KBr) ν_{max} : 3438–3383, 2930, 2847, 1625, 1594, 1556, 1503, 1355, 1216, 1202, 1124; ¹H NMR (CDCl₃, 300 MHz): δ 13.47 (1H, s, 2'-OH), 7.86 (1H, d, *J* = 15.3 Hz, H-β), 7.83 (1H, d, *J* = 9.4 Hz, H-6'), 7.62 (2 × 1H, d, *J* = 8.7 Hz, H-2,6), 7.46 (1H, d, *J* = 15.3 Hz, H-α), 7.46–7.33 (5 × 1H, m, 4-OBn), 7.02 (2 × 1H, d, *J* = 8.7 Hz, H-3,5), 6.44 (1H, dd, *J* = 9.4, 2.4 Hz, H-5'), 6.42 (1H, s, H-3'), 5.88 (1H, br s, 4'-OH), 5.13 (2H, s, 4-OCH₂); ¹³C NMR (CDCl₃, 125 MHz): δ 191.9 (C=O), 166.4 (C-2'), 162.5 (C-4'), 161.0 (C-4), 146.4 (C-β), 136.3 (C1-Bn), 131.8 (C-6'), 130.4 (C-2,6),128.7 (C3,5-Bn), 128.2 (C4-Bn), 127.7 (C-1), 127.5 (C2,6-Bn),117.9 (C-α), 115.3 (C-3,5), 114.6 (C-1'), 107.6 (C-5'), 103.7 (C-3'), 70.1 (4-OCH₂); ESI-HRMS (+) *m*/*z*: Anal. Calcd for C₂₂H₁₉O₄ (M+H)⁺: 347.1278; found: 347.1276.

4.1.9. 4-Chloro-2',4'-dihydroxychalcone (8)

Yield: 24%; ¹H NMR (CDCl₃, 300 MHz): δ 13.29 (1H, s, 2'-OH), 7.84 (1H, d, *J* = 15.9 Hz, H- β), 7.83 (1H, d, *J* = 7.5 Hz, H-6'), 7.62– 7.57 (2 × 1H, m, H-2,6), 7.54 (1H, d, *J* = 15.9 Hz, H- α), 7.42–7.30 (2 × 1H, m, H-3,5), 6.49–6.43 (1H, m, H-5'), 6.43 (1H, s, H-3'), 5.74 (1H, br s, 4'-OH); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₅H₁₂ClO₃ (M+H)⁺: 275.0470; found: 275.0469.

4.1.10. 2,4-Dichloro-2',4'-dihydroxychalcone (9)

Yield: 3%; 1H NMR (CDCl₃, 500 MHz): δ 13.20 (1H, s, 2'-OH), 8.19 (1H, d, *J* = 15.5 Hz, H-β), 7.80 (1H, d, *J* = 9.5 Hz, H-6'), 7.69 (1H, d, *J* = 8.5 Hz, H-6), 7.54 (1H, d, *J* = 15.5 Hz, H-α), 7.44 (1H, d, *J* = 2.1 Hz, H-3), 7.32 (1H, dd, *J* = 8.5, 2.1 Hz, H-5), 6.44 (1H, dd, *J* = 9.5, 2.5 Hz, H-5'), 6.43 (1H, s,H-3'); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₅H₁₁Cl₂O₃ (M+H)⁺: 309.0080; found: 309.0079.

4.1.11. 2',4'-Dihydroxy-4-methoxychalcone (10)

Yield: 54%; ¹H NMR (CDCl₃, 300 MHz): δ 13.47 (1H, s, 2'-OH), 7.87 (1H, d, *J* = 15.1 Hz, H-β), 7.84 (1H, d, *J* = 9.6 Hz, H-6'), 7.62 (2 × 1H, d, *J* = 8.8 Hz, H-2,6), 7.46 (1H, d, *J* = 15.1 Hz, H-α), 6.95 (2 × 1H, d, *J* = 8.8 Hz, H-3,5), 6.44 (1H, dd, *J* = 9.6, 2.2 Hz, H-5'), 6.42 (1H, s, H-3'), 5.71 (1H, br s, 4'-OH), 3.87 (3H, s, 4-OCH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₆H₁₅O₄ (M+H)⁺: 271.0965; found: 271.0964.

4.1.12. 4-Fluoro-2',4'-dihydroxychalcone (11)

Yield: 10% as yellow solid; mp: 178–180 °C; IR (KBr) v_{max} : 3438, 2912, 2846, 2730, 1630, 1592, 1550, 1500, 1353, 1311, 1279, 1221, 1140; ¹H NMR (CDCl₃, 300 MHz): δ 13.38 (1H, s, 2'-OH), 7.86 (1H, d, *J* = 15.5 Hz, H-β), 7.83 (1H, d, *J* = 9.7 Hz, H-6'), 7.65 (2 × 1H, d, *J* = 8.6 Hz, H-2,6), 7.50 (1H, d, *J* = 15.5 Hz, H-α), 7.13 (2 × 1H, d, *J* = 8.6 Hz, H-3,5), 6.45 (1H, dd, *J* = 9.7, 2.5 Hz, H-5'), 6.43 (1H, s, H-3'), 5.75 (1H, br s, 4'-OH); ¹³C NMR (CDCl₃, 125 MHz): δ 191.7 (C=O), 166.5 (C-2'), 162.7 (C-4'), 143.2 (C-β), 131.9 (C-6'), 132.6 (C-1), 131.0 (C-4), 130.5 (C-2,6), 120.0 (C-α), 116.2 (C-3,5), 114.4 (C-1'), 107.7 (C-5'), 103.8 (C-3'); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₅H₁₂FO₃ (M+H)⁺: 259.0765; found: 259.0764.

4.1.13. 2',4'-Dihydroxy-2,4-bis(methoxymethoxy)-3'-propylchalcone (12)

Yield: 12% as yellow solid; mp: 158–160 °C; IR (KBr) v_{max} : 3439, 2952, 2926, 2866, 1614, 1542, 1474, 1437, 1370, 1311, 1293, 1236, 1154, 1107; ¹H NMR (CDCl₃, 500 MHz): *δ* 13.75 (1H, s, 2'-OH), 8.12 $(1H, d, J = 15.6 \text{ Hz}, H-\beta)$, 7.63 (1H, d, J = 8.8 Hz, H-6'), 7.54 (1H, d, J)I = 8.7 Hz, H-6, 7.52 (1H, d, $I = 15.6 \text{ Hz}, \text{ H-}\alpha$), 6.79 (1H, d, *J* = 2.3 Hz, H-3), 6.68 (1H, dd, *J* = 8.7, 2.3 Hz, H-5), 6.33 (1H, d, I = 8.8 Hz, H-5'), 5.21 (2H, s, 2-OCH₂), 5.14 (2H, s, 4-OCH₂), 3.45 (3H, s, 2-OCH₃), 3.43 (3H, s, 4-OCH₃), 2.59 (1H, t, *J* = 7.7 Hz, 3'-CH₂CH₂CH₃), 1.57-1.51 (1H, m, 3'-CH₂CH₂CH₃), 0.93 (1H, t, I = 7.4 Hz, 3'-CH₂CH₂CH₃); ¹³C NMR (CDCl₃, 125 MHz): δ 192.6 (C=O), 164.4 (C-2'), 160.5 (C-4'), 159.9 (C-4), 157.8 (C-2), 139.3 (C-β), 129.8 (C-6), 128.8 (C-6'), 118.9 (C-α), 118.5 (C-1), 116.0 (C-3'), 114.3 (C-1'), 109.4 (C-5), 106.9 (C-5'), 103.3 (C-3), 94.7 (2-OCH₂), 94.3 (4-OCH₂), 56.5 (2-OCH₃), 56.3 (4-OCH₃), 24.5 (3'-CH₂CH₂CH₃), 21.8 (3'-CH₂CH₂CH₃), 14.2 (3'-CH₂CH₂CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₂₂H₂₇O₇ (M+H)⁺: 403.1751; found: 403.1749.

4.1.14. 2',4'-Dihydroxy-3,4,5-trimethoxy-3'-propylchalcone (13)

Yield: 34% as yellow solid; mp: 165–167 °C; IR (KBr) ν_{max} : 3215–3170, 2944, 2917, 2859, 2826, 1702, 1621, 1569, 1542, 1494, 1440, 1411, 1362, 1318, 1274, 1212, 1148, 1120, 1098; ¹H NMR (CDCl₃, 300 MHz): δ 13.68 (1H, s, 2'-OH), 7.80 (1H, d, J = 15.4 Hz, H-β), 7.72 (1H, d, J = 8.9 Hz, H-6'), 7.46 (1H, d, J = 15.4 Hz, H-α), 6.86 (2 × 1H, s, H-2,6), 6.43 (1H, d, J = 8.9 Hz, H-5'), 5.78 (1H, br s, 4'-OH), 3.93 (2 × 3H, s, 3,5-OCH₃), 3.91 (3H, s, 4-OCH₃), 2.67 (2H, t, J = 7.7 Hz, 3'-CH₂CH₂CH₃), 1.68–1.56 (2H, m, 3'-CH₂CH₂CH₃), 1.00 (3H, t, J = 7.4 Hz, 3'-CH₂CH₂CH₃); ¹³C NMR (CDCl₃, 125 MHz): δ 192.0 (C=O), 164.5 (C-2'), 160.5 (C-4'), 153.4 (C-3,5), 144.3 (C-β), 140.4 (C-4), 130.4 (C-1), 128.9 (C-6'), 119.7 (C-α), 116.3 (C-3'), 114.1 (C-1'), 107.2 (C-5'), 105.7 (C-2,6), 61.0 (4-OCH₃), 56.2 (3,5-OCH₃), 24.5 (3'-CH₂CH₂CH₃), 21.8 (3'-CH₂CH₂CH₃), 14.2 (3'-CH₂CH₂CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₂₁H₂₅O₆ (M+H)⁺: 313.1646; found: 313.1643.

4.1.15. 2',4'-Dihydroxy-2,4-dimethoxy-3'-propylchalcone (14)

Yield: 51%; ¹H NMR (CDCl₃, 300 MHz): δ 13.89 (1H, s, 2'-OH), 8.12 (1H, d, *J* = 15.5 Hz, H- β), 7.70 (1H, d, *J* = 8.9 Hz, H-6'), 7.62 (1H, d, *J* = 15.5 Hz, H- α), 7.57 (1H, d, *J* = 8.5 Hz, H-6), 6.58 (1H, dd, *J* = 8.5, 2.4 Hz, H-5), 6.48 (1H, d, *J* = 2.4 Hz, H-3), 6.40 (1H, d, *J* = 8.9 Hz, H-5'), 3.92 (3H, s, 2-OCH₃), 3.87 (3H, s, 4-OCH₃), 2.68– 2.61 (2H, m, 3'-CH₂CH₂CH₃), 1.63–1.58 (2H, m, 3'-CH₂CH₂CH₃); 1.02–0.92 (3H, m, 3'-CH₂CH₂CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₂₀H₂₃O₅ (M+H)⁺: 343.1540; found: 343.1538.

4.1.16. 2',4'-Dihydroxy-2,4,5-trimethoxy-3'-propylchalcone (15)

Yield: 28% as orange solid; mp: 180–183 °C; IR (KBr) ν_{max} : 3397, 2940, 2902, 2844, 1602, 1540, 1498, 1448, 1422, 1364, 1273, 1222, 1195, 1093; ¹H NMR (CDCl₃, 300 MHz): δ 13.89 (1H, s, 2'-OH), 8.17 (1H, d, *J* = 15.5 Hz, H-β), 7.72 (1H, d, *J* = 8.9 Hz, H-6'), 7.55 (1H, d, *J* = 15.5 Hz, H-α), 7.12 (1H, s, H-6), 6.53 (1H, s, H-3), 6.41 (1H, d, *J* = 8.9 Hz, H-5'), 5.44 (1H, br s, 4'-OH), 3.96 (3H, s, 2-OCH₃), 3.93 (3H, s, 5-OCH₃), 3.92 (3H, s, 4-OCH₃), 2.66 (2H, t, *J* = 7.7 Hz, 3'-CH₂CH₂CH₃), 1.66–1.56 (2H, m, 3'-CH₂CH₂CH₃); 1.00 (3H, t, *J* = 7.4 Hz, 3'-CH₂CH₂CH₃); ¹³C NMR (CDCl₃, 125 MHz): δ 192.6 (C=O), 164.4 (C-2'), 159.8 (C-4'), 154.8 (C-2), 154.6 (C-5), 143.2 (C-4), 139.6 (C-β), 128.8 (C-6'), 118.3 (C-α), 116.0 (C-3'), 115.4 (C-1), 114.3 (C-1'), 111.7 (C-6), 106.8 (C-5'), 96.7 (C-3), 56.6 (4-OCH₃), 56.3 (5-OCH₃), 56.1 (2-OCH₃), 24.5 (3'-CH₂CH₂CH₃), 21.9 (3'-CH₂CH₂CH₃), 14.2 (3'-CH₂CH₂CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₂₁H₂₅O₆ (M+H)⁺: 373.1646; found: 373.1643.

4.1.17. 4-Bromo-2',4'-dihydroxy-3'-propylchalcone (16)

Yield: 11%; ¹H NMR (CDCl₃, 300 MHz): δ 13.58 (1H, s, 2'-OH), 7.80 (1H, d, *J* = 15.6 Hz, H- β), 7.73 (1H, d, *J* = 8.7 Hz, H-6'), 7.54– 7.44 (2 × 1H, m, H-2,6), 7.34–7.26 (2 × 1H, m, H-3,5), 7.57 (1H, d, *J* = 15.6 Hz, H- α), 6.45 (1H, d, *J* = 8.7 Hz, H-5'), 5.77 (1H, br s, 4'-OH), 2.59–2.55 (2H, m, 3'-CH₂CH₂CH₃), 1.65–1.57 (2H, m, 3'-CH₂CH₂CH₃), 1.02–0.93 (3H, m, 3'-CH₂CH₂CH₃); ESI-HRMS (+) *m*/ *z*: Anal. Calcd for C₁₈H₁₈BrO₃ (M+H)⁺: 361.0434; found: 361.0432.

4.1.18. 2',4'-Dihydroxy-3'-propyl-4-trifluoromethylchalcone (17)

Yield: 4%; ¹H NMR (CDCl₃, 300 MHz): δ 13.53 (1H, s, 2'-OH), 7.88 (1H, d, *J* = 15.6 Hz, H- β), 7.75 (2 × 1H, d, *J* = 8.4 Hz, H-2,6), 7.71 (1H, d, *J* = 9.0 Hz, H-6'), 7.68 (2 × 1H, d, *J* = 8.4 Hz, H-3,5), 7.65 (1H, d, *J* = 15.6 Hz, H- α), 6.43 (1H, d, *J* = 9.0 Hz, H-5'), 2.69– 2.64 (2H, m, 3'-CH₂CH₂CH₃), 1.68–1.58 (2H, m, 3'-CH₂CH₂CH₃), 1.00 (3H, t, *J* = 7.4 Hz, 3'-CH₂CH₂CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₉H₁₈F₃O₃ (M+H)⁺: 351.1203; found: 351.1201.

4.1.19. 4-Chloro-2',4'-dihydroxy-3'-propylchalcone (18)

Yield: 12%; ¹H NMR (CDCl₃, 300 MHz): δ 7.84 (1H, d, *J* = 15.5 Hz, H-β), 7.70 (1H, d, *J* = 8.8 Hz, H-6'), 7.58 (2 × 1H, d, *J* = 8.5 Hz, H-2,6), 7.56 (1H, d, *J* = 15.5 Hz, H-α), 7.41–7.26 (2 × 1H, m, H-3,5), 6.42 (1H, d, *J* = 8.8 Hz, H-5'), 2.62–2.55 (2H, m, 3'-CH₂CH₂CH₃), 1.65–1.57 (2H, m, 3'-CH₂CH₂CH₃), 1.02–0.93 (3H, m, 3'-CH₂CH₂-CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₈H₁₈ClO₃ (M+H)⁺: 317.0939; found: 317.0938.

4.1.20. 2',4'-Dihydroxy-4-methoxy-3'-propylchalcone (19)

Yield: 26% as yellow solid; mp: 177–180 °C; IR (KBr) ν_{max} : 3355, 2945, 2924, 2851, 1618, 1594, 1542, 1501, 1476, 1413, 1359, 1279, 1226, 1196, 1161, 1096; ¹H NMR (CDCl₃, 300 MHz): δ 13.78 (1H, s, 2'-OH), 7.86 (1H, d, *J* = 15.4 Hz, H- β), 7.72 (1H, d, *J* = 8.9 Hz, H-6'), 7.61 (2 × 1H, d, *J* = 8.8 Hz, H-2,6), 7.24 (1H, d, *J* = 15.4 Hz, H- α), 6.95 (2 × 1H, d, *J* = 8.8 Hz, H-3,5), 6.41 (1H, d, *J* = 8.9 Hz, H-5'), 5.39 (1H, br s, 4'-OH), 3.87 (3H, s, 4-OCH₃), 2.66 (2H, t, *J* = 7.7 Hz, 3'-CH₂CH₂CH₃), 1.65–1.58 (2H, m, 3'-CH₂CH₂CH₃), 1.00 (3H, t, *J* = 7.4 Hz, 3'-CH₂CH₂CH₃); ¹³C NMR (CDCl₃, 125 MHz): δ 192.2 (C=O), 164.4 (C-2'), 161.7 (C-4), 160.0 (C-4'), 144.1 (C- β), 130.3

(C-2–6), 128.8 (C-6'), 127.6 (C-1), 118.0 (C-α), 116.1 (C-3'), 114.4 (C-3,5), 114.2 (C-1'), 107.0 (C-5'), 55.4 (4-OCH₃), 24.4 (3'-CH₂CH₂CH₃), 21.8 (3'-CH₂CH₂CH₃), 14.2 (3'-CH₂CH₂CH₃); ESI-HRMS (+) m/z: Anal. Calcd for C₁₉H₂₁O₄ (M+H)⁺: 313.1434; found: 313.1435.

4.1.21. 4-Fluoro-2',4'-dihydroxy-3'-propylchalcone (20)

Yield: 4%; ¹H NMR (CDCl₃, 300 MHz): δ 13.64 (1H, s, 2'-OH), 7.85 (1H, d, *J* = 15.5 Hz, H-β), 7.71 (1H, d, *J* = 8.9 Hz, H-6'), 7.64 (2 × 1H, d, *J* = 8.7 Hz, H-2,6), 7.52 (1H, d, *J* = 15.5 Hz, H-α), 7.14 (2 × 1H, d, *J* = 8.7 Hz, H-3,5), 6.42 (1H, d, *J* = 8.9 Hz, H-5'), 5.48 (1H, br s, 4'-OH), 2.66 (2H, t, *J* = 7.7 Hz, 3'-CH₂CH₂CH₃), 1.65–1.59 (2H, m, 3'-CH₂CH₂CH₃), 1.03–0.95 (3H, m, 3'-CH₂CH₂CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₈H₁₈FO₃ (M+H)⁺: 301.1235; found: 301.1233.

4.1.22. 2-Chloro-2',4'-dihydroxy-3'-propylchalcone (21)

Yield: 11% as yellow solid; mp: 184–188 °C; IR (KBr) ν_{max} : 3461–3389, 2940, 2911, 2842, 1611, 1544, 1474, 1449, 1424, 1351, 1295, 1258, 1221, 1100; ¹H NMR (CDCl₃, 300 MHz): δ 13.56 (1H, s, 2'-OH), 8.26 (1H, d, *J* = 15.5 Hz, H-β), 7.70 (1H, d, *J* = 8.9 Hz, H-6'), 7.77–7.73 (1H, m, H-3), 7.58 (1H, d, *J* = 15.5 Hz, H-α), 7.47–7.44 (1H, m, H-6), 7.35–7.32 (2 × 1H, m, H-4,5), 6.42 (1H, d, *J* = 8.9 Hz, H-5'), 5.51 (1H, br s, 4'-OH), 2.66 (2H, t, *J* = 7.7 Hz, 3'-CH₂CH₂CH₃), 1.65–1.58 (2H, m, 3'-CH₂CH₂CH₃), 1.00 (3H, t, *J* = 7.4 Hz, 3'-CH₂CH₂CH₃); ¹³C NMR (CDCl₃, 125 MHz): δ 191.9 (C=O), 164.2 (C-2'), 160.4 (C-4'), 140.0 (C-β), 135.5 (C-1), 133.2 (C-2), 131.2 (C-4), 130.4 (C-6), 129.0 (C-6'), 127.9 (C-3), 127.0 (C-5), 123.3 (C-α), 116.2 (C-3'), 114.1 (C-1'), 107.3 (C-5'), 24.4 (3'-CH₂CH₂CH₃), 21.8 (3'-CH₂CH₂CH₃), 14.2 (3'-CH₂CH₂CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₈H₁₈ClO₃ (M+H)⁺: 317.0939; found: 317.0938.

4.1.23. 2',4'-Dihydroxy-3'-methyl-2,4-bis(methoxymethoxy)chalcone (22)

Yield: 15% as yellow solid; mp: 150–153 °C; IR (KBr) ν_{max} : 3460–3403, 2907, 2839, 1711, 1602, 1530, 1484, 1439, 1362, 1285, 1218, 1138, 1098, 1057, 972; ¹H NMR (CDCl₃, 300 MHz): *δ* 13.89 (1H, s, 2'-OH), 8.20 (1H, d, *J* = 15.6 Hz, H-β), 7.71 (1H, d, *J* = 8.9 Hz, H-6'), 7.62 (1H, d, *J* = 8.7 Hz, H-6), 7.59 (1H, d, *J* = 15.6 Hz, H-α), 6.87 (1H, d, *J* = 2.3 Hz, H-3), 6.75 (1H, dd, *J* = 8.7 Hz, H-5), 6.42 (1H, d, *J* = 8.9 Hz, H-5'), 5.29 (2H, s, 2-OCH₂), 5.21 (2H, s, 4-OCH₂), 3.52 (3H, s, 2-OCH₃), 3.50 (3H, s, 4-OCH₃), 2.17 (3H, s, 3'-CH₃); ¹³C NMR (CDCl₃, 125 MHz): *δ* 192.6 (C=O), 164.3 (C-2'), 160.5 (C-4'), 159.9 (C-4), 157.9 (C-2), 139.4 (C-β), 129.8 (C-6), 128.6 (C-6'), 118.9 (C-α), 118.5 (C-1), 114.1 (C-1'), 110.0 (C-3'), 109.4 (C-5), 106.7 (C-5'), 103.3 (C-3), 94.7 (2-OCH₂), 94.3 (4-OCH₂), 56.5 (2-OCH₃), 56.3 (4-OCH₃), 7.4 (3'-CH₃); ESI-HRMS *m/z*: Anal. Calcd for C₂₀H₂₃O₇ (M+H)⁺: 375.1438; found: 375.1436.

4.1.24. 2',4'-Dihydroxy-3'-methyl-3,4,5-trimethoxychalcone (23)

Yield: 3%; ¹H NMR (CDCl₃, 300 MHz): δ 13.73 (1H, s, 2'-OH), 7.82 (1H, d, *J* = 15.4 Hz, H- β), 7.73 (1H, d, *J* = 8.9 Hz, H-6'), 7.47 (1H, d, *J* = 15.4 Hz, H- α), 6.87 (2 × 1H, s, H-2,6), 6.44 (1H, d, *J* = 8.9 Hz, H-5'), 3.94 (2 × 3H, s, 3,5-OCH₃), 3.91 (3H, s, 4-OCH₃), 2.18 (3H, s, 3'-CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₉H₂₁O₆(M+H)⁺: 345.1333; found: 345.1332.

4.1.25. 2',4'-Dihydroxy-3'-methyl-2,4,5-trimethoxychalcone (24)

Yield: 36%; ¹H NMR (CDCl₃, 300 MHz): δ 13.95 (1H, s, 2'-OH), 8.18 (1H, d, *J* = 15.5 Hz, H- β), 7.75 (1H, d, *J* = 8.7 Hz, H-6'), 7.55 (1H, d, *J* = 15.5 Hz, H- α), 7.12 (1H, s, H-6), 6.54 (1H, s, H-3), 6.42 (1H, d, J = 8.7 Hz, H-5'), 5.55 (1H, br s, 4'-OH), 3.96 (3H, s, 2-OCH₃), 3.93 (3H, s, 5-OCH₃), 3.92 (3H, s, 4-OCH₃), 2.17 (3H, s, 3'-CH₃); ESI-HRMS (+) m/z: Anal. Calcd for $C_{19}H_{21}O_6(M+H)^+$: 345.1333; found: 345.1331.

4.1.26. 4-Bromo-2',4'-dihydroxy-3'-methylchalcone (25)

Yield: 26%; ¹H NMR (CDCl₃, 300 MHz): δ 13.64 (1H, s, 2'-OH), 7.82 (1H, d, *J* = 15.6 Hz, H-β), 7.72 (1H, d, *J* = 8.9 Hz, H-6'), 7.59 (1H, d, *J* = 15.6 Hz, H-α), 7.55–7.46 (2 × 1H, m, H-2,6), 7.32 (2 × 1H, d, *J* = 8.6 Hz, H-3,5), 6.44 (1H, d, *J* = 8.9 Hz, H-5'), 5.49 (1H, br s, 4'-OH), 2.18 (3H, s, 3'-CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₆H₁₄BrO₃(M+H)⁺: 333.0121; found: 333.0119.

4.1.27. 2',4'-Dihydroxy-3'-methyl-4-trifluoromethylchalcone (26)

Yield: 6% as yellow solid; mp: 164–168 °C; IR (KBr) v_{max} : 3422–3393, 2909, 2843, 1691, 1621, 1551, 1473, 1426, 1354, 1309, 1279, 1224, 1162, 1095, 1052; ¹H NMR (CDCl₃, 300 MHz): δ 13.55 (1H, s, 2'-OH), 7.88 (1H, d, *J* = 15.5 Hz, H- β), 7.75 (2 × 1H, d, *J* = 8.6 Hz, H-2,6), 7.71 (1H, d, *J* = 9.3 Hz, H-6'), 7.68 (2 × 1H, d, *J* = 8.6 Hz, H-3,5), 7.65 (1H, d, *J* = 15.5 Hz, H- α), 6.45 (1H, d, *J* = 9.3 Hz, H-5'), 5.50 (1H, br s, 4'-OH), 2.18 (3H, s, 3'-CH₃); ¹³C NMR (CDCl₃, 75 MHz): δ 191.5 (C=O), 164.5 (C-2'), 160.0 (C-4'), 142.2 (C- β), 138.9 (C-4), 132.4 (C-1), 128.8 (C-6'), 128.5 (C-2,6), 125.9 (C-3,5), 125.9 (4-CF₃), 122.8 (C- α), 113.9 (C-1'), 111.5 (C-3'), 107.2 (C-5'), 7.4 (3'-CH₃) ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₇H₁₄F₃O₃ (M+H)⁺: 323.0890; found: 323.0888.

4.1.28. 4-Benzyloxy-2',4'-dihydroxy-3'-methylchalcone (27)

Yield: 26%; ¹H NMR (CDCl₃, 300 MHz): δ 13.81 (1H, s, 2'-OH), 7.87 (1H, d, *J* = 15.4 Hz, H- β), 7.72 (1H, d, *J* = 8.9 Hz, H-6'), 7.62 (2 × 1H, d, *J* = 8.8 Hz, H-2,6), 7.48 (1H, d, *J* = 15.4 Hz, H- α), 7.43– 7.33 (5H, m, 4-OBn), 7.02 (2 × 1H, d, *J* = 8.8 Hz, H-3,5), 6.42 (1H, d, *J* = 8.9 Hz, H-5'), 5.36 (1H, br s, 4'-OH), 5.13 (2H, s, 4-OCH₂), 2.18 (3H, s, 3'-CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₂₃H₂₁O₄(M+H)⁺: 361.1434; found: 361.1432.

4.1.29. 4-Chloro-2',4'-dihydroxy-3'-methylchalcone (28)

Yield: 9% as yellowsolid; mp: 204–207 °C; IR (KBr) v_{max} : 3442, 2923, 2862, 1709, 1633, 1553, 1487, 1441, 1367, 1317, 1239, 1107; ¹H NMR (CDCl₃, 300 MHz): δ 13.63 (1H, s, 2'-OH), 7.83 (1H, d, *J* = 15.5 Hz, H-β), 7.71 (1H, d, *J* = 8.8 Hz, H-6'), 7.59 (2 × 1H, d, *J* = 8.5 Hz, H-2,6), 7.56 (1H, d, *J* = 15.5 Hz, H-α), 7.40 (2 × 1H, d, *J* = 8.5 Hz, H-3,5), 6.43 (1H, d, *J* = 8.8 Hz, H-5'), 5.41 (1H, br s, 4'-OH), 2.17 (3H, s, 3'-CH₃); ¹³C NMR (CDCl₃, 125 MHz): δ 191.5 (C=O), 163.9 (C-2'), 162.4 (C-4'), 142.1 (C-β), 136.1 (C-1), 133.3 (C-4), 129.4 (C-2,6), 129.1 (C-3,5), 128.5 (C-6'), 121.2 (C-α), 113.0 (C-1'), 111.8 (C-3'), 107.1 (C-5'), 7.2 (3'-CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₆H₁₄ClO₃(M+H)⁺: 289.0626; found: 289.0624.

4.1.30. 2,4-Dichloro-2',4'-dihydroxy-3'-methylchalcone (29)

Yield: 6% as yellowsolid; mp: 194–197 °C; IR (KBr) v_{max} : 3462– 3399, 2923, 2845, 1623, 1480, 1456, 1432, 1356, 1303, 1252, 1225, 1204, 1099; ¹H NMR (CDCl₃, 300 MHz): δ 13.54 (1H, s, 2'-OH), 8.18 (1H, d, *J* = 15.5 Hz, H-β), 7.69 (1H, d, *J* = 8.4 Hz, H-6), 7.68 (1H, d, *J* = 8.9 Hz, H-6'), 7.56 (1H, d, *J* = 15.5 Hz, H-α), 7.48 (1H, d, *J* = 2.1 Hz, H-3), 7.31 (1H, dd, *J* = 8.4, 2.1 Hz, H-5), 6.43 (1H, d, *J* = 8.9 Hz, H-5'), 5.46 (1H, br s, 4'-OH), 2.17 (3H, s, 3'-CH₃); ¹³C NMR (CDCl₃, 125 MHz): δ 191.5 (C=0), 164.5 (C-2'), 160.5 (C-4'), 138.8 (C-β), 136.5 (C-4), 136.1 (C-2), 131.8 (C-1),130.2 (C-3), 128.8 (C-6'), 128.6 (C-6),127.5 (C-5), 123.5 (C-α), 114.0 (C-1'), 111.5 (C-3'), 107.1 (C-5'), 7.3 (3'-CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₆H₁₃Cl₂O₃ (M+H)⁺: 323.0236; found: 323.0235.

4.1.31. 2',4'-Dihydroxy-3'-methyl-4-methoxychalcone (30)

Yield: 69%; ¹H NMR (CDCl₃, 300 MHz): *δ* 13.81 (1H, s, 2'-OH), 7.87 (1H, d, *J* = 15.4 Hz, H-β), 7.72 (1H, d, *J* = 8.9 Hz, H-6'), 7.62 (2 × 1H, d, *J* = 8.7 Hz, H-2,6), 7.48 (1H, d, *J* = 15.4 Hz, H-α), 6.95 (2 × 1H, d, *J* = 8.7 Hz, H-3,5), 6.42 (1H, d, *J* = 8.9 Hz, H-5'), 5.41 (1H, br s, 4'-OH), 3.87 (3H, s, 4-OCH₃), 2.18 (3H, s, 3'-CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for $C_{17}H_{17}O_4$ (M+H)⁺: 285.1121; found: 285.1120.

4.1.32. 4-Fluoro-2',4'-dihydroxy-3'-methylchalcone (31)

Yield: 34%; ¹H NMR (CDCl₃, 300 MHz): *δ* 13.67 (1H, s, 2'-OH), 7.85 (1H, d, *J* = 15.5 Hz, H-β), 7.71 (1H, d, *J* = 8.9 Hz, H-6'), 7.64 (2 × 1H, d, *J* = 8.7 Hz, H-2,6), 7.52 (1H, d, *J* = 15.5 Hz, H-α), 7.14 (2 × 1H, d, *J* = 8.7 Hz, H-3,5), 6.34 (1H, d, *J* = 8.9 Hz, H-5'), 5.47 (1H, br s, 4'-OH), 2.17 (3H, s, 3'-CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₆H₁₄FO₃ (M+H)⁺: 273.0922; found: 273.0920.

4.1.33. 7-Hydroxy-2',4',5'-trimethoxy-8-propylflavanone (32)

Yield: 5% as yellow solid; mp: 186–189 °C; IR (KBr) ν_{max} : 3422, 2930, 2902, 2833, 1640, 1563, 1496, 1417, 1266, 1187, 1081, 1011; ¹H NMR (CDCl₃, 300 MHz): δ 7.75 (1H, d, *J* = 8.6 Hz, H-5), 7.20 (1H, s, H-6'), 6.56 (1H, s, H-3'), 6.53 (1H, d, *J* = 8.6 Hz, H-6), 5.74 (1H, dd, *J* = 12.9, 3.4 Hz, H-2), 3.94 (3H, s, 2'-OCH₃), 3.90 (3H, s, 4'-OCH₃), 3.83 (3H, s, 5'-OCH₃), 2.92 (1H, dd, *J* = 16.9, 3.4 Hz, H-3_{eq}), 2.81 (1H, dd, *J* = 16.9, 12.9 Hz, H-3_{ax}), 2.69–2.63 (2H, m, 8-CH₂CH₂CH₂OH₃), 1.67–1.58 (2H, m, 8-CH₂CH₂CH₃), 0.97 (3H, t, *J* = 7.4 Hz, 8-CH₂CH₂CH₃); ¹³C NMR (CDCl₃, 125 MHz): δ 192.1 (C-4), 161.7 (C-7), 159.9 (C-8a), 150.1 (C-5'), 149.4 (C-2'), 143.1 (C-4'), 126.1 (C-5), 119.3 (C-1'), 116.3 (C-8), 115.2 (C-4a), 110.0 (C-6'), 109.8 (C-6), 97.0 (C-3'), 74.6 (C-2), 56.5 (4'-OCH₃), 56.2 (2'-OCH₃), 56.1 (5'-OCH₃), 43.5 (C-3), 24.9 (8-CH₂CH₂CH₃), 22.2 (8-CH₂CH₂CH₃), 14.1 (8-CH₂CH₂CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₂₁H₂₅O₆ (M+H)⁺: 373.1646; found: 373.1645.

4.1.34. 7-Hydroxy-8-methyl-2',4'-dimethoxyflavanone (33)

Yield: 56% as yellow solid; mp: 206–209 °C; IR (KBr) ν_{max} : 3422–3147, 2907, 2815, 1695, 1595, 1528, 1489, 1445, 1414, 1351, 1305, 1281, 1256, 1199, 1143, 1088, 1058, 1010; ¹H NMR (CDCl₃, 300 MHz): δ 7.74 (1H, d, *J* = 8.7 Hz, H-5), 7.53 (1H, d, *J* = 8.5 Hz, H-6'), 6.58 (1H, dd, *J* = 8.5, 2.3 Hz, H-5'), 6.52 (1H, dd, *J* = 8.7 Hz, H-6), 6.50 (1H, d, *J* = 2.3 Hz, H-3'), 5.73 (1H, dd, *J* = 11.3, 4.7 Hz, H-2), 5.48 (1H, br s, 7-OH), 3.84 (3H, s, 2'-OCH₃), 3.82 (3H, s, 4'-OCH₃), 2.92–2.78 (2H, m, H-3), 2.15 (3H, s, 8-CH₃); ¹³C NMR (CDCl₃, 125 MHz): δ 192.4 (C-4), 161.8 (C-7), 160.8 (C-2'), 160.1 (C-8a), 157.1 (C-4'), 127.0 (C-6'), 125.9 (C-5), 120.4 (C-1'), 115.0 (C-4a), 111.6 (C-8), 109.4 (C-6), 104.3 (C-5'), 98.4 (C-3'), 74.6 (C-2), 55.4 (2'-OCH₃), 55.3 (4'-OCH₃), 43.3 (C-3), 7.9 (8-CH₃); ESI-HRMS (+) *m/z*: Anal. Calcd for C₁₈H₁₉O₅ (M+H)⁺: 315.1227; found: 315.1225.

4.2. Biological activity

4.2.1. Reagents and stock solutions of compounds for biological assays

Fetal bovine serum (FBS), phosphate buffer saline (PBS), and trypsin were purchased from Gibco Invitrogen Co. (Scotland, UK). RPMI-1640 medium with ultraglutamine was purchased from Lonza (Basel, Switzerland). Dimethylsulfoxide (DMSO), doxorubicin, ethylenediaminetetracetic acid (EDTA), propidium iodide (PI), RNase, sulforhodamine B (SRB) and trypan blue were purchased from SigmaChemical Co. (Saint Louis, MO, USA). Trichloroacetic acid (TCA) and Tris were purchased from Merck (Darmstadt, Germany). Stock solutions of tested compounds were prepared in DMSO and stored at -20 °C. Appropriate dilutions of the compounds were freshly prepared in medium just prior to the assays. Final concentrations of DMSO did not interfere with the biological activity tested.

4.2.2. Cell culture

The following 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). All cell lines were grown as monolayer and routinely maintained in RPMI-1640 medium with ultraglutamine supplemented with 5% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO₂.

4.2.3. Growth inhibition assay

Cells were plated in 96-well plates at appropriate densities in order to ensure exponential growth throughout the experimental period $(5.0 \times 10^3 \text{ cells/well}$ for MCF-7 and NCI-H460 and $7.5 \times 10^3 \text{ cells/well}$ for A375-C5) and then allowed to adhere overnight. Cells were then treated for 48 h with five serial dilutions of each compound (1:2 or 1:3). Following this incubation period, cells were fixed in situ with trichloroacetic acid, washed and stained with SRB.^{17,20} The bound stain was then solubilised with Tris and the absorbance was measured at 492 nm in a plate reader (Biotek Instruments Inc. PowerWave XS, Winooski, USA). The effect of the vehicle solvent (DMSO) on the growth of these cell lines was evaluated by exposing untreated control cells to the maximum concentration of DMSO used in each assay (0.25%).

4.2.4. Cell cycle analysis

MCF-7 cells were plated in 6-well plates $(5.0 \times 10^3 \text{ cells/well})$ and incubated at 37 °C for 24 h. Exponentially growing cells were then incubated with the compounds (2, 7, 11, 13, 15, 21 and 29) at their respective GI₅₀ concentrations (previously determined with the SRB assay, 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% ethanol at 4 °C for at least 12 h and subsequently resuspended in PBS containing 0.1 mg/mL RNase A and 5 µg/mL propidium iodide (PI). Cellular DNA content, for cell cycle distribution analysis, was measured by flow cytometry using an Epics XL-MCL Coulter flow cytometer (Brea, CA, USA) plotting at least 20,000 events *per* sample, as previously described.^{21,22} The percentage of cells in the G1, S and G2/M phases of the cell cycle and the percentage of cells in the sub-G1 peak were determined using the FlowJo 7.2 software (Tree Star, Inc., Ashland, OR, USA) after cell debris exclusion.

4.2.5. Analysis of cellular apoptosis

MCF-7 cells were incubated with the compounds as described above. Cells were then harvested and the Human Annexin-V-FITC/PI apoptosis kit (Bender MedSystems, Vienna, Austria) was used according to the manufacturer's instructions in order to detect apoptotic cells. Flow cytometry was carried out using 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 using the FlowJo 7.2 software (Tree Star, Inc., Ashland, OR, USA), as previously described.^{23,24}

4.2.6. Statistical analysis

All experimental data are presented as means ± SEM from at least three independent experiments (most of them performed in duplicate). Statistical analysis was carried out using an unpaired student's *t*-test. All analysis were performed comparing cells treated with compounds with blank cells (cells incubated with medium only). * Indicates $p \leq 0.05$ and ** $p \leq 0.01$.

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