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# Synthesis and cytotoxic, anti-inflammatory, and anti-oxidant activities of 2′,5′-dialkoxylchalcones as cancer chemopreventive agents

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ABSTRACT

In an effort to develop novel anti-tumor, or cancer chemopreventive agents, a series of 2′,5′-dialkoxylchalcones were prepared by Claisen–Schmidt condensation of appropriate acetophenones with suitable aromatic aldehyde. In vitro screening revealed low micromolar activity ($IC_{50}$) against several human cancer cell lines. Selective compound **10** induced an accumulation of A549 cells in the $G_2$/M phase arrest which was well correlated with inhibitory activity against tubulin polymerization. Cytotoxic compounds **3** and **12** showed significant inhibitory effects on NO production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophage-like cells while cytotoxic compound **10** revealed potent inhibitory effect on TNF-α formation in RAW 264.7 cells in response to LPS. Compounds **3** and **10** also showed significant inhibitory effects on xanthine oxidase. The present results suggested that compounds **3** and **10** were potential to be served as cancer chemopreventive agents.



## 1. Introduction

Chalcones are characterized with diverse biological activities, among which anti-inflammatory, anti-malaria, anti-protozoal, anti-bacterial, nitric oxide inhibition, tyrosinase inhibition, cytotoxic, anticancer, and anti-leishmanial activities have been reported in the past decade.[1–5] In our previous papers, we have demonstrated that various synthetic chalcones were potential cancer chemopreventive agents.[6,7]

Mast cells, neutrophils and macrophages play important roles in inflammatory disorders. Nitric oxide (NO) accumulation from macrophages induced cytotoxicity and expressed NO may lead to pathophysiology of septic shock.[8] The excess production of NO also can destroy functional normal tissues during acute and chronic inflammation related mechanistically to carcinogenesis.[9] Among the synthetic chalcone derivatives in our previous paper, 2′,5′-dimethoxy-4-hydroxychalcone showed potent inhibitory effect on NO accumulation from RAW 264.7 cells and potent selective cytotoxicity against human MCF-7 cells and caused cell death by apoptosis.[7] 2′,5′-Dimethoxy-2-(5-methylthienyl)chalcone was also suggested to be a significant $G_2$/M arrest-mediated apoptosis-inducing agent and inhibitor of NO production in rat macrophages.[7]

Reactive oxygen species (ROS) is implicated in numerous pathological events including inflammation, metabolic disorders, cellular aging, reperfusion damage, artherosclerosis, and carcinogenesis.[10] The oxidative damage of DNA induced by ROS lead to certain cancers. Xanthine oxidase (XO) is a key enzyme that catalyzes the oxidation of xanthine and hypoxanthine into uric acid and plays a vital role in causing hyperuricemia and gout.[11] In the investigation on anti-oxidant agent, we found that synthetic chalcones were significantly inhibited oxidative damage of DNA and XO activity. For continual discovery of potential cancer chemopreventive agents with cytotoxic, anti-inflammatory and anti-oxidant activities, we reported the synthesis, cytotoxicity, anti-inflammatory activity and anti-oxidant activity of a series of 2′,5′-dialkoxyl and related chalcones in the present paper.

## 2. Results and discussion

### 2.1. Chemistry

As shown in Scheme 1, new chalcones, **1**, **9**, **13**, and **14** were prepared by using Claisen–Schmidt condensation of appropriate

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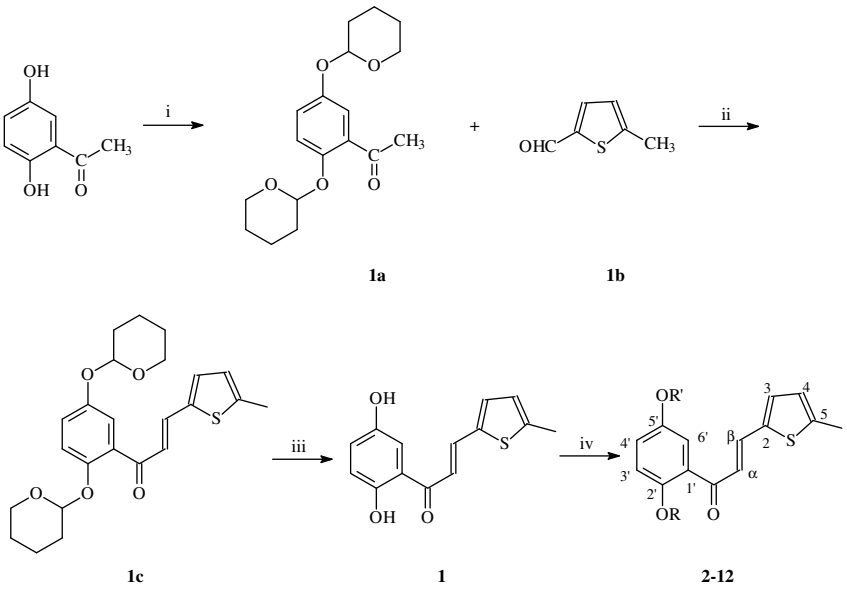


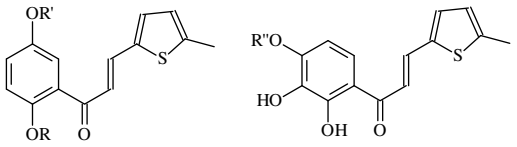

**Scheme 1.** Reagents and conditions: (i) Pyridinium *p*-toluenesulfonate, 3,4-dihydro-α-pyran, $CH_2Cl_2$, rt, 4 h; (ii) $BaOH \cdot 8H_2O$, MeOH, 40 °C; (iii) *p*-toluenesulfonic acid, rt, 4 h, 5% $NaHCO_3$; (iv) alkyl halides, $K_2CO_3$, acetone, rt.

hydroxyacetophenones, protected as tetrahydropyranyl ether, with appropriate aromatic aldehyde.[12] A mixture of **1**, appropriate alkyl halide, and potassium carbonate in appropriate solvent were stirred at room temperature to afford new chalcones, **2**–**8** and **10**–**12**. These procedures afforded various chalcone derivatives in a good yield (Table 1).

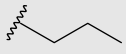
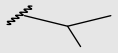
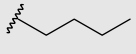
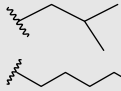
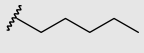
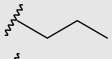
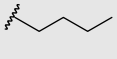
## 2.2. Biological results and discussion

The MTT microassay was used for the evaluation of cytotoxic properties of the synthetic compounds in this research. The growth-inhibitory effects was undertaken in four human cell lines, including MCF-7 (breast), A549 (lung), Hep3B (liver) and HT-29

**Table 1**
Structure and analytical data of chalcones




**1-12** **13,14**

| Compound | R | R′ | R″ | Formula | Mp (°C) | Yield% | Analysis[a] |
|---|---|---|---|---|---|---|---|
| **1** | H | H | | $C_{14}H_{12}O_3S$ | 184–185 | 58 | C, H, S |
| **2** | H | $CH_2CH_3$ | | $C_{16}H_{16}O_3S$ | 99–100 | 89 | C, H, S |
| **3** | $CH_2CH_3$ | $CH_2CH_3$ | | $C_{18}H_{20}O_3S$ | 68–69 | 77 | C, H, S |
| **4** | H |  | | $C_{17}H_{18}O_3S$ | 83–84 | 85 | C, H, S |
| **5** | H |  | | $C_{17}H_{18}O_3S$ | 100–101 | 79 | C, H, S |
| **6** | H |  | | $C_{18}H_{20}O_3S$ | 86–87 | 75 | C, H, S |
| **7** | H |  | | $C_{18}H_{20}O_3S$ | 99–100 | 64 | C, H, S |
| **8** | H |  | | $C_{19}H_{22}O_3S$ | 82–83 | 68 | C, H, S |
| **9** | H | $CH_3$ | | $C_{15}H_{14}O_3S$ | 78–79 | 55 | C, H, S |
| **10** | $CH_2CH_3$ | $CH_3$ | | $C_{17}H_{18}O_3S$ | 70–71 | 71 | C, H, S |
| **11** |  | $CH_3$ | | $C_{18}H_{20}O_3S$ | Oil | 65 | C, H, S |
| **12** |  | $CH_3$ | | $C_{19}H_{22}O_3S$ | Oil | 53 | C, H, S |
| **13** | | | $CH_3$ | $C_{15}H_{14}O_4S$ | 131–132 | 48 | C, H, S |
| **14** | | | H | $C_{14}H_{12}O_4S$ | 182–183 | 46 | C, H, S |

[a] C, H and S analyses were within ±0.4% of the theoretical values.

(colorectal) followed the method described previously.[13] The results are listed in Table 2. All compounds in Table 2 showed selective cytotoxicity against MCF-7 cells except for compound **10**. In our previous report, we have definitely indicated that 5-methylthiophene substituted at B ring of 2′,5′-dimethoxychalcone significantly enhanced the cytotoxicity.[7] In this study, we founded that the different alkoxyl groups and hydroxy groups substituted at both C-2′ and C-5′ leaded to different cytotoxic effects. Compounds **2** and **4**–**9** with hydroxyl group at C-2′ and various alkoxyl groups at C-5′ showed different cytotoxic effect against several human cancer cell lines used in Table 2. The increasing length of alkoxyl branched side chains at C-5′ significantly enhanced cytotoxic activities against MCF-7 and HT-29 cells, while both C-2′ and C-5′ were substituted with ethoxy groups, such as compound **3**, revealed more stronger cytotoxic effects than that of **2**, especially for cytotoxicity against MCF-7 cells. Compound **10**, a 2′-ethoxy-5′-methoxy chalcone derivative revealed potent cytotoxicity against the four human cancer cell lines used in Table 2. We previously reported 2′,5′-dimethoxy-2-(5-methylthienyl)chalcone, **15**, showed potent cytotoxicity against A 549, Hep3B, HT-29 and MCH-7 cells.[7] When the methoxyl group substituted at C-2′ of **15** replaced by ethoxyl group, such as **10**, significantly enhanced the cytotoxicity against MCF-7, Hep3B, and HT-29 cells.

**Table 2**
Cytotoxicity of **2**–**14** ($IC_{50}$ values in μM)[a]

| Compound | Cell line | | | |
|---|---|---|---|---|
| | MCF-7 | A549 | Hep3B | HT-29 |
| **2** | 23.2 ± 2.1 | — | 90.2 ± 2.0 | 43.4 ± 1.2 |
| **3** | 1.4 ± 1.9 | 4.4 ± 2.6 | 31.6 ± 2.3 | 3.2 ± 2.0 |
| **4** | 21.5 ± 2.1 | — | 23.2 ± 2.3 | 125.7 ± 2.0 |
| **5** | 15.5 ± 2.1 | — | 81.7 ± 2.0 | 30.2 ± 0.5 |
| **6** | 38.9 ± 1.2 | — | 91.7 ± 1.2 | 71.4 ± 0.9 |
| **7** | 13.0 ± 2.2 | — | 54.4 ± 0.5 | 30.7 ± 0.6 |
| **8** | 48.4 ± 2.2 | — | 112.0 ± 1.2 | 86.9 ± 2.1 |
| **9** | 34.6 ± 2.1 | — | 87.5 ± 0.9 | 71.5 ± 1.5 |
| **10** | 2.3 ± 2.1 | 6.3 ± 2.4 | 0.2 ± 0.1 | 0.1 ± 0.3 |
| **11** | 0.8 ± 0.3 | — | 7.0 ± 0.1 | 86.6 ± 0.3 |
| **12** | 13.9 ± 1.6 | — | 10.7 ± 1.5 | 61.1 ± 2.3 |
| **13** | 20.7 ± 1.5 | — | 10.7 ± 2.1 | 53.4 ± 2.3 |
| **14** | — | — | — | 35.9 ± 0.5 |
| 5-Fu | 1.5 ± 0.1 | 3.1 ± 0.2 | 0.6 ± 0.3 | 0.6 ± 0.1 |

[a] Data are presented as means ± SEM ($n = 6$). Compounds **2**–**14** or 5-Fu dissolved in DMSO, were diluted with culture medium containing 0.1% DMSO, respectively. The control cells were treated with culture medium containing 0.1% DMSO. 5-Fu (5-fluorouracil) was used as a positive control. —, not determined.

Whereas cell proliferation and differentiation are specifically controlled in the $G_1$ phase and the $G_1$-S transition in the cell cycle, oncogenic progress exert their greatest effect by targeting particular regulators of $G_1$ phase progression.[14] To determine the phase of the cell cycle of human A549 cells at which selective compound **10** exerts its growth-inhibitory effect, cells were treated with different concentration of **10** for 24 h and analyzed by flow cytometry (Fig. 1 and Table 3). In the cell cycle of **10**, $G_2$/M phase arrest was apparent after treatment with 10 μM of **10**. This indicated that the $G_2$/M phase arrest of **10** is probably induced by inhibition of tubulin polymerization, which is also correlated with cell growth inhibition.[15] These results demonstrated that this series may be a anti-mitotic agent inhibiting tubulin polymerization with anti-proliferative activity.

Selective compounds **3**, **10**, **11**, **12**, and **13** with significant cytotoxicity against several human cancer cells were studied in vitro for their inhibitory effects on chemical mediators released from mast cells, neutrophils and macrophages. These compounds had no significant inhibitory effects on the release of β-glucuronidase from rat mast cells stimulated with compound 48/80 (data not shown), the release of β-glucuronidase and lysozyme from neutrophils stimulated with fromyl-Met-Leu-Phe (fMLP)/cytochalasin B (CB) (data not shown) and superoxide anion generation in rat neu-

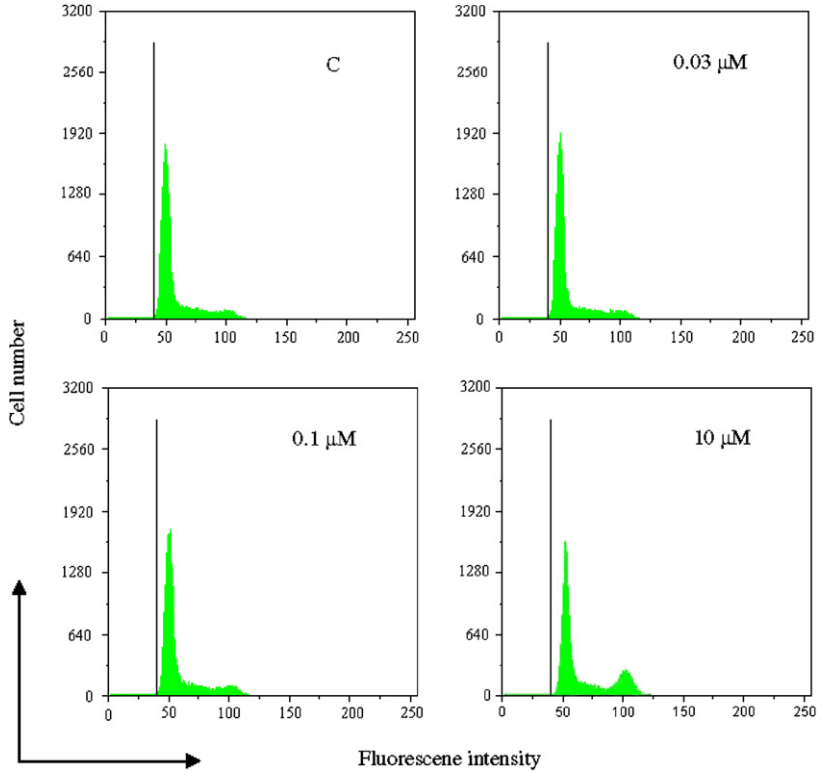



**Figure 1.** Flow cytometric analysis of the DNA histograms of propidium iodide (PI)-stained A549 cells. The cells were incubated with various concentrations of **10** for 24 h. After incubation, the cells were fixed and incubated with PI and RNase before reading red fluorescence exited by blue light. The control (C) cells were treated without **10**.

**Table 3**
Cell populations of cell cycle of A549 cells treated with various concentration of **10**

| | $G_1$ % | S % | $G_2$/M % |
|---|---|---|---|
| C | 67.857 | 30.683 | 1.46 |
| **10** (0.03 μM) | 70.207 | 27.69 | 2.104 |
| **10** (0.1 μM) | 65.919 | 28.384 | 5.698 |
| **10** (10 μM) | 64.102 | 24.271 | 11.627 |

The cell populations of cell cycle were analyzed by LYSIS II software. The control (C) cells were treated with medium.

trophils stimulated with fMLP/CB or phorbol myristate (PMA) (data not shown). Treatment of RAW 264.7 macrophage-like cells with LPS for 24 h induced NO production as assessed by measuring the accumulation of nitrite, a stable metabolite of NO in the media, based on Griess.[16] As shown in Figure 2, compounds **3** and **12** showed potent and concentration-dependent inhibitory effects on NO accumulation from 264.7 cells with $IC_{50}$ values of 16.5 ± 1.5 and 28.0 ± 4.2 μM, respectively. NO plays a central role in macrophage-induced cytotoxicity and expressed NO may contribute to the pathophysiology of septic shock.[8] NO with excessive production also lead to destruction of functional normal tissues during acute and chronic inflammation.[9] Effect on the generation of tumor necrosis factor-α (TNF-α) was determined in RAW 264.7 cells activated by LPS.[17–19] As shown in Figure 3, compound **10** showed significantly inhibitory effects on the generation of TNF-α in RAW 264.7 cells activated by LPS with an $IC_{50}$ value of 0.4 ± 0.2 μM. It indicated a stronger inhibitory effect on the generation of TNF-α than that of positive control (genistein) and 2′-ethoxy and 5′-methoxy groups substituted on the A ring revealed significant inhibition of TNF-α in RAW 264.7 cells. The strong inhibition of TNF-α and potent cytotoxic activity against the cell lines used in Table 2 of **10** suggested that **10** may be a potential cancer chemopreventive agent.

The anti-oxidant efficiency of free radical scavenging activities, oxidase inhibition and inhibition of oxidative DNA damage were studied in vitro by agarose gel electrophoresis.[20] As shown in Figure 4, selective compounds, **3** and **10**, showed protective effects of oxidative DNA damage caused by $O_2^{-}\cdot$ generated by xanthine (XA)/xanthine oxidase (XO). Compounds **3** and **10** each at 300 μM showed protective effect on oxidative DNA damage caused by $O_2^{-}\cdot$. The above results clearly indicated that **3** and **10** possessed 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity or inhibitory effect on XO. For determination of the anti-oxidant activity of **3** and **10**, the radical scavenging activity and XO inhibitory activity of **3** and **10** were analyzed. As shown at Figure 5, compound **3** and allopurinol (positive control) significantly inhibit the XO activity in a concentration-dependent manner with $IC_{50}$ values of 21.3 ± 15.3 and 2.0 ± 0.7 μM, respectively, while compound **10** weakly inhibit

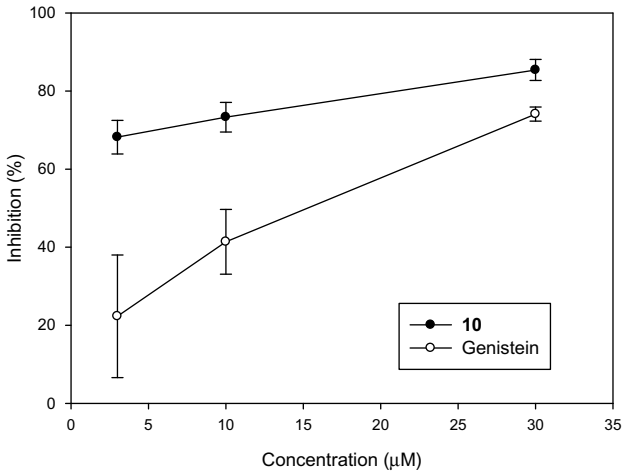



**Figure 3.** The inhibitory effects of **10** on the accumulation of TNF-α formation in the culture media of RAW 264.7 cells in response to LPS (1 μg/mL). Data are presented as mean ± SD ($n$ = 3). Genistein was used as a positive control.

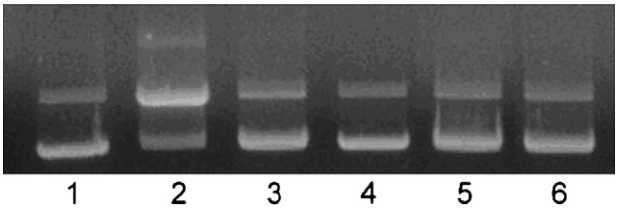



**Figure 4.** Inhibition of DNA strand breaks induced by $O_2^{-}\cdot$ (generated by XA/XO) in the presence of **3** and **10** studied by gel electrophoresis. Supercoiled plasmid pBR322 DNA (500 ng) in phosphate buffer (pH 7.4) solution was incubated for 20 min with XA/XO acting as the control. Lane 1, DNA (without XA/XO); lane 2, control; lane 3, control + SOD (300 μM); lane 4, control + quercetin (300 μM) serving as positive control; lane 5, control + **3** (300 μM); lane 6, control + **10** (300 μM).

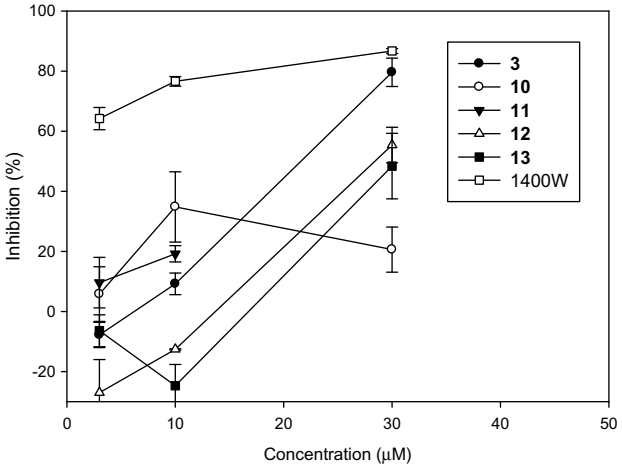



**Figure 2.** The inhibitory effects of **3** and **10**–**13** on the accumulation of $NO_2^{-}$ formation in the culture media of RAW 264.7 cells in response to LPS (1 μg/mL). Data are presented as mean ± SD ($n$ = 3). *N*-(3-Aminomethyl)benzylacetamidine (1400 W) was used as a positive control.

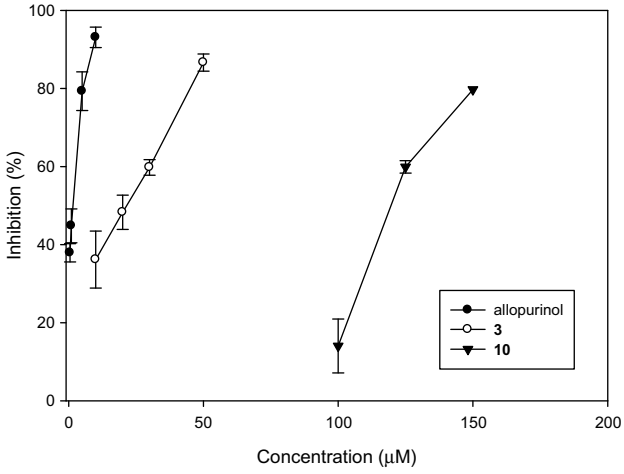



**Figure 5.** Dose-dependent inhibition of XO by **3**, **10**, and allopurinol. Data are presented as means ± SEM, $n$ = 3–6.

the XO activity also in a concentration-dependent manner with an $IC_{50}$ value of 131.0 ± 10.9 μM. Compounds **3** and **10** did not show DPPH scavenging activity.

## 3. Conclusion

In this study, we have synthesized a series of new compounds of 2′,5′-dialkoxy-2-(5-methylthienyl)chalcone and studied on their biological activities of cytotoxicity, anti-inflammation, anti-oxidation, and xanthine oxidase inhibitory effect. This series of compounds revealed cytotoxicity against human cancer cells. Among them, compounds **3** and **10** showed potent cytotoxicity against a several human cancer cells, potent activity on the inhibition of oxidative DNA damage, potent anti-inflammatory activity, and anti-oxidant effect. The present research suggested that compounds **3** and **10** may be used as cancer chemopreventive agents.

## 4. Experimental

### 4.1. Chemistry

Melting points (uncorrected) were determined with a Yanco micro melting point apparatus. IR spectra were determined with a Perkin-Elmer system 2000 FTIR spectrophotometer. $^1H$ (400 MHz) and $^{13}C$ (100 MHz) NMR spectra were recorded on a Varian UNITY-400 spectrometer, and mass spectra were obtained on a JMX-HX 100 mass spectrometer. Elemental analyses were within ± 0.4% of the theoretical values, unless otherwise noted. Chromatography was performed using a flash-column technique on silica gel 60 supplied by E. Merck.

### 4.2. Synthesis of chalcones

#### 4.2.1. 2′,5′-Dihydroxy-2-(5-methylthienyl)chalcone (1)

2′,5′-Dihydroxyacetophenone (3.8 g, 25 mmol), 3,4-dihydro-α-pyran (13 mL) and pyridinium *p*-toluenesulfonate (0.15 g, 0.6 mmol) were reacted in $CH_2Cl_2$ (100 mL) to give 2′,5′-di(tetrahydropyran-2-yloxy)acetophenone (**1a**). Compound **1a** (8.0 g, 25 mmol), 5-methylthiophene-2-aldehyde (3.2 g, 25 mmol) and barium hydroxide octahydrate (4.29 g, 25 mmol) were refluxed in methanol (100 mL) and then the solvent was evaporated under reduced pressure. The residue and *p*-toluenesulfonic acid (0.1 g, 0.6 mmol) in methanol (100 mL) was stirred for 4 h at room temperature, and then evaporated in vacuo. Water was added to the mixture, neutralized with 5% $NaHCO_3$ (50 mL) and extracted with EtOAc. The organic layer was separated, washed with $H_2O$, dried and evaporated in vacuo. The residue was eluted through a silica gel column with *n*-hexane/EtOAc, 4:1 to afford **1** (3.77 g, 14.5 mmol, 58%) as red crystals, mp 184–185 °C. IR (KBr) 3442, 1635, 1569 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): $\delta$ 2.60 (3H, s, $CH_3$), 6.78 (1H, dd, $J$ = 3.6, 0.8 Hz, H-3), 6.92 (1H, d, $J$ = 8.8 Hz, H-3′), 7.04 (1H, dd, $J$ = 8.8, 2.8 Hz, H-4′), 7.21 (1H, d, $J$ = 3.6 Hz, H-4), 7.21 (1H, d, $J$ = 14.8 Hz, H-α), 7.32 (1H, d, $J$ = 2.8 Hz, H-6′), 7.96 (1H, d, $J$ = 14.8 Hz, H-β), 12.46 (1H, s, OH). $^{13}C$ NMR ($CDCl_3$): $\delta$ 16.0 ($CH_3$), 114.4 (C-3′), 117.3 (C-4′), 119.3 (C-α), 119.8 (C-1′), 124.6 (C-3), 127.1 (C-6′), 133.9 (C-4), 138.2 (C-2), 138.6 (C-β), 145.7 (C-5), 147.2 (C-5′), 157.8 (C-2′), 192.6 (C═O). EIMS (70 eV) *m*/*z* (% rel. int.): 260 (46) $[M]^+$.

#### 4.2.2. General procedure for obtaining chalcones 2–8, 10–12

Appropriate **1**, alkyl iodide and potassium carbonate were reacted in acetone at room temperature. The solvent was evaporated under reduced pressure. The residue was dissolved in 10% HCl and extracted with EtOAc. The organic layer was separated, washed with $H_2O$, dried and evaporated in vacuo. The residue was chromatographed over a silica gel column and eluted with appropriate solvent to yield compounds **2–8**, **10–12**.

#### 4.2.3. 5′-Ethoxy-2′-hydroxy-2-(5-methylthienyl)chalcone (2)

Compound **1** (2.95 g, 10.2 mmol), ethyl iodide (1.59 g, 10.2 mmol) and potassium carbonate (1.41 g, 10.2 mmol) were treated as in general method for the synthesis of chalcone to afford **2** (2.62 g, 9.1 mmol, 89%) as orange crystals, mp 99–100 °C. IR (KBr) 3446, 1646, 1570 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): $\delta$ 1.44 (3H, t, $J$ = 6.8 Hz, $CH_3$), 2.55 (3H, s, $CH_3$), 4.05 (2H, q, $J$ = 7.2 Hz, $OCH_2$), 6.78 (1H, dd, $J$ = 3.6, 1.2 Hz, H-3), 6.95 (1H, d, $J$ = 8.8 Hz, H-3′), 7.12 (1H, dd, $J$ = 8.8, 2.8 Hz, H-4′), 7.21 (1H, d, $J$ = 3.6 Hz, H-4), 7.23 (1H, d, $J$ = 15.2 Hz, H-α), 7.32 (1H, d, $J$ = 2.8 Hz, H-6′), 7.97 (1H, d, $J$ = 15.2 Hz, H-β), 12.47 (1H, s, OH). $^{13}C$ NMR ($CDCl_3$): $\delta$ 14.9 ($CH_3$), 16.0 ($CH_3$), 64.5 ($OCH_2$), 114.0 (C-6′), 117.4 (C-3′), 119.1 (C-α), 119.7 (C-1′), 124.0 (C-3), 127.1 (C-4′), 133.8 (C-4), 138.2 (C-2), 138.4 (C-β), 145.6 (C-5), 150.9 (C-2′), 157.7 (C-5′), 192.8 (C═O). EIMS (70 eV) *m*/*z* (% rel. int.): 288 (39) $[M]^+$.

#### 4.2.4. 2′,5′-Diethoxy-2-(5-methylthienyl)chalcone (3)

Compound **1** (2.95 g, 10.2 mmol), ethyl iodide (3.18 g, 20.4 mmol) and potassium carbonate (1.41 g, 10.2 mmol) were treated as in general method for the synthesis of chalcone to afford **3** (2.50 g, 7.9 mmol, 77%) as yellow crystals, mp 68–69 °C. IR (KBr) 1641, 1565 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): $\delta$ 1.39 (3H, t, $J$ = 7.2 Hz, $CH_3$), 1.42 (3H, t, $J$ = 6.8 Hz, $CH_3$), 2.51 (3H, s, $CH_3$), 4.02 (2H, q, $J$ = 7.2 Hz, $OCH_2$), 4.07 (2H, q, $J$ = 7.2 Hz, $OCH_2$), 6.72 (1H, dd, $J$ = 3.6, 1.2 Hz, H-3), 6.90 (1H, d, $J$ = 8.8 Hz, H-3′), 7.00 (1H, dd, $J$ = 8.8, 3.2 Hz, H-4′), 7.10 (1H, d, $J$ = 3.6 Hz, H-4), 7.23 (1H, d, $J$ = 3.2 Hz, H-6′), 7.25 (1H, d, $J$ = 15.6 Hz, H-α), 7.71 (1H, d, $J$ = 15.2 Hz, H-β). $^{13}C$ NMR ($CDCl_3$): $\delta$ 14.9 ($CH_3$), 15.0 ($CH_3$), 15.9 ($CH_3$), 64.1 ($OCH_2$), 65.1 ($OCH_2$), 114.6 (C-6′), 115.0 (C-3′), 120.2 (C-α), 124.9 (C-3), 126.7 (C-4′), 129.6 (C-1′), 132.4 (C-4), 135.7 (C-β), 138.9 (C-2), 144.0 (C-5), 152.1 (C-2′), 152.9 (C-5′), 191.4 (C═O). EIMS (70 eV) *m*/*z* (% rel. int.): 316 (57) $[M]^+$.

#### 4.2.5. 2′-Hydroxy-5′-propoxy-2-(5-methylthienyl)chalcone (4)

Compound **1** (2.95 g, 10.2 mmol), normal propyl iodide (1.73 g, 10.2 mmol) and potassium carbonate (1.41 g, 10.2 mmol) were treated as in general method for the synthesis of chalcone to afford **4** (2.63 g, 8.7 mmol, 85%) as orange crystals, mp 83–84 °C. IR (KBr) 3446, 1641, 1570 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): $\delta$ 1.07 (3H, t, $J$ = 7.2 Hz, $CH_3$), 1.83 (2H, m, $CH_2$), 2.55 (3H, s, $CH_3$), 3.93 (2H, t, $J$ = 6.4 Hz, $OCH_2$), 6.78 (1H, dd, $J$ = 3.6, 1.2 Hz, H-3), 6.95 (1H, d, $J$ = 9.2 Hz, H-3′), 7.12 (1H, dd, $J$ = 9.2, 3.2 Hz, H-4′), 7.21 (1H, d, $J$ = 3.6 Hz, H-4), 7.25 (1H, d, $J$ = 15.2 Hz, H-α), 7.31 (1H, d, $J$ = 3.2 Hz, H-6′), 7.97 (1H, d, $J$ = 15.2 Hz, H-β), 12.47 (1H, s, OH). $^{13}C$ NMR ($CDCl_3$): $\delta$ 10.5 ($CH_3$), 16.0 ($CH_3$), 22.7 ($CH_2$), 70.6 ($OCH_2$), 113.9 (C-6′), 117.4 (C-3′), 119.1 (C-α), 119.7 (C-1′), 124.1 (C-3), 127.1 (C-4′), 133.7 (C-4), 138.2 (C-2), 138.4 (C-β), 145.6 (C-5), 151.1 (C-2′), 157.7 (C-5′), 192.8 (C═O). EIMS (70 eV) *m*/*z* (% rel. int.): 302 (70) $[M]^+$.

#### 4.2.6. 2′-Hydroxy-5′-iso-propoxy-2-(5-methylthienyl)chalcone (5)

Compound **1** (2.95 g, 10.2 mmol), isopropyl iodide (1.73 g, 10.2 mmol) and potassium carbonate (1.41 g, 10.2 mmol) were treated as in general method for the synthesis of chalcone to afford **5** (2.44 g, 8.1 mmol, 79%) as orange crystals, mp 100–101 °C. IR (KBr) 3446, 1637, 1570 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): $\delta$ 1.35 (6H, d, $J$ = 6.0 Hz, 2 × $CH_3$), 2.55 (3H, s, $CH_3$), 4.47 (1H, m,OCH), 6.78 (1H, dd, $J$ = 3.6, 1.2 Hz, H-3), 6.94 (1H, d, $J$ = 8.8 Hz, H-3′), 7.12 (1H, dd, $J$ = 8.8, 2.8 Hz, H-4′), 7.21 (1H, d, $J$ = 3.6 Hz, H-4), 7.31 (1H, d, $J$ = 15.2 Hz, H-α), 7.35 (1H, d, $J$ = 2.8 Hz, H-6′), 7.96 (1H, d, $J$ = 15.2 Hz, H-β), 12.47 (1H, s, OH). $^{13}C$ NMR ($CDCl_3$): $\delta$ 16.0 ($CH_3$), 22.1 (2 × $CH_3$), 71.7 (OCH), 116.8 (C-6′), 117.4 (C-3′), 119.1

(C-α), 119.9 (C-1′), 125.7 (C-3), 127.1 (C-4′), 133.8 (C-4), 138.2 (C-2), 138.4 (C-β), 145.6 (C-5), 149.6 (C-2′), 157.9 (C-5′), 192.8 (C=O). EIMS (70 eV) *m*/*z* (% rel. int.): 302 (45) $[M]^+$.

#### 4.2.7. 5′-Butoxy-2′-hydroxy-2-(5-methylthienyl)chalcone (6)

Compound **1** (2.95 g, 10.2 mmol), normal butyl iodide (1.88 g, 10.2 mmol) and potassium carbonate (1.41 g, 10.2 mmol) were treated as in general method for the synthesis of chalcone to afford **6** (2.42 g, 7.7 mmol, 75%) as red crystals, mp 86–87 °C. IR (KBr) 3446, 1628, 1559 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): δ 1.00 (3H, t, *J* = 7.2 Hz, $CH_3$), 1.52 (2H, m, $CH_2$), 1.77 (2H, m, $CH_2$), 2.54 (3H, s, $CH_3$), 3.97 (2H, t, *J* = 6.4 Hz, $OCH_2$), 6.78 (1H, dd, *J* = 3.6, 1.2 Hz, H-3), 6.94 (1H, d, *J* = 9.2 Hz, H-3′), 7.12 (1H, dd, *J* = 9.2, 3.2 Hz, H-4′), 7.21 (1H, d, *J* = 3.6 Hz, H-4), 7.28 (1H, d, *J* = 15.2 Hz, H-α), 7.31 (1H, d, *J* = 3.2 Hz, H-6′), 7.97 (1H, d, *J* = 15.2 Hz, H-β), 12.47 (1H, s, OH). $^{13}C$ NMR ($CDCl_3$): δ 13.9 ($CH_3$), 16.0 ($CH_3$), 19.2 ($CH_2$), 31.4 ($CH_2$), 68.8 ($OCH_2$), 113.8 (C-6′), 117.4 (C-3′), 119.1 (C-α), 119.7 (C-1′), 124.0 (C-3), 127.1 (C-4′), 133.8 (C-4), 138.2 (C-2), 138.4 (C-β), 145.6 (C-5), 151.1 (C-2′), 157.7 (C-5′), 192.8 (C=O). EIMS (70 eV) *m*/*z* (% rel. int.): 316 (42) $[M]^+$.

#### 4.2.8. 2′-Hydroxy-5′-(2-methylpropoxy)-2-(5-methylthienyl)-chalcone (7)

Compound **1** (2.95 g, 10.2 mmol), 2-methylpropyl iodide (1.88 g, 10.2 mmol) and potassium carbonate (1.41 g, 10.2 mmol) were treated as in general method for the synthesis of chalcone to afford **7** (2.07 g, 6.5 mmol, 64%) as red crystals, mp 99–100 °C. IR (KBr) 3446, 1635, 1569 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): δ 1.06 (6H, d, *J* = 6.8 Hz, 2 × $CH_3$), 2.10 (1H, m, CH), 2.55 (3H, s, $CH_3$), 3.74 (2H, d, *J* = 6.8 Hz, $OCH_2$), 6.78 (1H, dd, *J* = 3.6, 1.6 Hz, H-3), 6.95 (1H, d, *J* = 8.8 Hz, H-3′), 7.12 (1H, dd, *J* = 8.8, 2.8 Hz, H-4′), 7.21 (1H, d, *J* = 3.6 Hz, H-4), 7.25 (1H, d, *J* = 15.6 Hz, H-α), 7.35 (1H, d, *J* = 2.8 Hz, H-6′), 7.97 (1H, d, *J* = 15.6 Hz, H-β), 12.46 (1H, s, OH). $^{13}C$ NMR ($CDCl_3$): δ 16.0 ($CH_3$), 19.3 (2 × $CH_3$), 28.4 (CH), 75.6 ($OCH_2$), 113.8 (C-6′), 117.5 (C-3′), 119.1 (C-α), 119.7 (C-1′), 124.2 (C-3), 127.1 (C-4′), 133.7 (C-4), 138.2 (C-2), 138.4 (C-β), 145.6 (C-5), 151.3 (C-2′), 157.7 (C-5′), 192.8 (C=O). EIMS (70 eV) *m*/*z* (% rel. int.): 316 (30) $[M]^+$.

#### 4.2.9. 2′-Hydroxy-5′-pentanoxy-2-(5-methylthienyl)chalcone (8)

Compound **1** (2.95 g, 10.2 mmol), normal pentanyl iodide (2.02 g, 10.2 mmol) and potassium carbonate (1.41 g, 10.2 mmol) were treated as in general method for the synthesis of chalcone to afford **8** (2.29 g, 6.9 mmol, 68%) as red crystals, mp 82–83 °C. IR (KBr) 3446, 1627, 1550 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): δ 0.95 (3H, t, *J* = 7.2 Hz, $CH_3$), 1.42 (4H, m, $CH_2$), 1.48 (2H, m, $CH_2$), 1.80 (2H, m, $CH_2$), 2.55 (3H, s, $CH_3$), 3.97 (2H, t, *J* = 6.4 Hz, $OCH_2$), 6.78 (1H, dd, *J* = 3.2, 1.2 Hz, H-3), 6.95 (1H, d, *J* = 8.8 Hz, H-3′), 7.12 (1H, dd, *J* = 8.8, 2.8 Hz, H-4′), 7.21 (1H, d, *J* = 3.2 Hz, H-4), 7.24 (1H, d, *J* = 15.2 Hz, H-α), 7.31 (1H, d, *J* = 2.8 Hz, H-6′), 7.97 (1H, d, *J* = 15.2 Hz, H-β), 12.46 (1H, s, OH). $^{13}C$ NMR ($CDCl_3$): δ 14.0 ($CH_3$), 16.0 ($CH_3$), 22.5 ($CH_2$), 28.2 ($CH_2$), 29.1 ($CH_2$), 69.1 ($OCH_2$), 113.9 (C-6′), 117.5 (C-3′), 119.1 (C-α), 119.7 (C-1′), 124.1 (C-3), 127.1 (C-4′), 133.7 (C-4), 138.2 (C-2), 138.4 (C-β), 145.5 (C-5), 151.2 (C-2′), 157.7 (C-5′), 192.8 (C=O). EIMS (70 eV) *m*/*z* (% rel. int.): 330 (63) $[M]^+$.

#### 4.2.10. 2′-Hydroxy-5′-methoxy-2-(5-methylthienyl)chalcone (9)

2′-Hydroxy-5′-methoxyacetophenone (4.2 g, 25 mmol), 3,4-dihydro-α-pyran (13 mL) and pyridinium *p*-toluenesulfonate (0.15 g, 0.6 mmol) were treated as in the method described in preparation of **1** to afford **9** (3.77 g, 13.8 mmol, 55%) as red crystals, mp 78–79 °C. IR (KBr) 3446, 1632, 1552 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): δ 2.53 (3H, s, $CH_3$), 3.83 (3H, s,$OCH_3$), 6.76 (1H, dd, *J* = 3.6, 0.8 Hz, H-3), 6.95 (1H, d, *J* = 8.8 Hz, H-3′), 7.12 (1H, dd, *J* = 8.8, 2.8 Hz, H-4′), 7.19 (1H, d, *J* = 3.6 Hz, H-4), 7.25 (1H, d, *J* = 15.2 Hz, H-α), 7.29 (1H, d, *J* = 2.8 Hz, H-6′), 7.95 (1H, d, *J* = 15.2 Hz, H-β), 12.49 (1H, s, OH). $^{13}C$ NMR ($CDCl_3$): δ 15.9 ($CH_3$), 56.0 ($OCH_3$), 112.7 (C-6′), 117.3 (C-3′), 119.2 (C-α), 119.6 (C-1′), 123.5 (C-3), 127.1 (C-4′), 133.7 (C-4), 138.1 (C-2), 138.4 (C-β), 145.6 (C-5), 151.6 (C-2′), 157.8 (C-5′), 192.7 (C=O). EIMS (70 eV) *m*/*z* (% rel. int.): 274 (18) $[M]^+$.

#### 4.2.11. 2′-Ethoxy-5′-methoxy-2-(5-methylthienyl)chalcone (10)

Compound **9** (2.88 g, 10.5 mmol), ethyl iodide (1.64 g, 10.5 mmol) and potassium carbonate (1.45 g, 10.5 mmol) were treated as in general method for the synthesis of chalcone to afford **10** (2.25 g, 7.5 mmol, 71%) as yellow crystal, mp 70–71 °C. IR (KBr) 1648, 1577 $cm^{-1}$;$^1H$ NMR ($CDCl_3$): δ 1.44 (3H, t, *J* = 6.8 Hz, $CH_3$), 2.50 (3H, s, $CH_3$), 3.80 (3H, s, $OCH_3$), 4.09 (2H, q, *J* = 6.8 Hz, $OCH_2$), 6.72 (1H, dd, *J* = 3.6, 1.2 Hz, H-3), 6.90 (1H, d, *J* = 8.8 Hz, H-3′), 7.00 (1H, dd, *J* = 8.8, 3.2 Hz, H-4′), 7.10 (1H, d, *J* = 3.6 Hz, H-4), 7.24 (1H, d, *J* = 3.2 Hz, H-6′), 7.26 (1H, d, *J* = 15.6 Hz, H-α), 7.72 (1H, d, *J* = 15.6 Hz, H-β). $^{13}C$ NMR ($CDCl_3$): δ 15.0 ($CH_3$), 15.8 ($CH_3$), 55.8 ($OCH_3$), 65.1 ($OCH_2$), 114.2 (C-6′), 114.6 (C-3′), 119.5 (C-α), 124.8 (C-3), 126.7 (C-4′), 129.6 (C-1′), 132.4 (C-4), 135.6 (C-β), 138.9 (C-2), 143.9 (C-5), 152.2 (C-2′), 153.5 (C-5′), 191.3 (C=O). EIMS (70 eV) *m*/*z* (% rel. int.): 302 (4) $[M]^+$.

#### 4.2.12. 5′-Methoxy-2′-propoxy-2-(5-methylthienyl)chalcone (11)

Compound **9** (2.88 g, 10.5 mmol), normal propyl iodide (1.78 g, 10.5 mmol) and potassium carbonate (1.45 g, 10.5 mmol) were treated as in general method for the synthesis of chalcone to afford **11** (2.16 g, 6.8 mmol, 65%) as orange oil. IR (KBr) 1650, 1573 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): δ 1.03 (3H, t, *J* = 7.2 Hz, $CH_3$), 1.83 (2H, m, $CH_2$), 2.50 (3H, s, $CH_3$), 3.80 (3 H, s, $OCH_3$), 3.96 (2H, t, *J* = 6.4 Hz, $OCH_2$), 6.72 (1H, dd, *J* = 3.6, 1.2 Hz, H-3), 6.90 (1H, d, *J* = 8.8 Hz, H-3′), 7.00 (1H, dd, *J* = 8.8, 2.8 Hz, H-4′), 7.10 (1H, d, *J* = 3.6 Hz, H-4), 7.23 (1H, d, *J* = 2.8 Hz, H-6′), 7.24 (1H, d, *J* = 15.6 Hz, H-α), 7.72 (1H, d, *J* = 15.6 Hz, H-β). $^{13}C$ NMR ($CDCl_3$): δ 10.8 ($CH_3$), 15.9 ($CH_3$), 22.7 ($CH_2$), 55.8 ($OCH_3$), 71.0 ($OCH_2$), 114.2 (C-6′), 114.3 (C-3′), 119.5 (C-α), 124.7 (C-3), 126.7 (C-4′), 129.6 (C-1′), 132.4 (C-4), 135.7 (C-β), 138.8 (C-2), 144.0 (C-5), 152.3 (C-2′), 153.4 (C-5′), 191.5 (C=O). EIMS (70 eV) *m*/*z* (% rel. int.): 316 (13) $[M]^+$.

#### 4.2.13. 2′-Butoxy-5′-methoxy-2-(5-methylthienyl)chalcone (12)

Compound **9** (2.88 g, 10.5 mmol), normal butyl iodide (1.93 g, 10.5 mmol) and potassium carbonate (1.45 g, 10.5 mmol) were treated as in general method for the synthesis of chalcone to afford **12** (1.84 g, 5.6 mmol, 53%) as orange oil. IR (KBr) 1644, 1531 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): δ 0.92 (3H, t, *J* = 7.2 Hz, $CH_3$), 1.49 (2H, m, $CH_2$), 1.79 (2H, m, $CH_2$), 2.50 (3H, s, $CH_3$), 3.80 (3H, s, $OCH_3$), 4.00 (2H, t, *J* = 6.4 Hz, $OCH_2$), 6.72 (1H, dd, *J* = 3.2, 1.2 Hz, H-3), 6.90 (1H, d, *J* = 8.8 Hz, H-3′), 7.00 (1H, dd, *J* = 8.8, 3.2 Hz, H-4′), 7.10 (1H, d, *J* = 3.2 Hz, H-4), 7.23 (1H, d, *J* = 3.2 Hz, H-6′), 7.24 (1H, d, *J* = 15.2 Hz, H-α), 7.72 (1H, d, *J* = 15.2 Hz, H-β). $^{13}C$ NMR ($CDCl_3$): δ 13.8 ($CH_3$), 15.8 ($CH_3$), 19.4 ($CH_2$), 31.4 ($CH_2$), 55.8 ($OCH_3$), 69.1 ($OCH_2$), 114.2 (C-6′), 114.3 (C-3′), 119.5 (C-α), 124.8 (C-3), 126.6 (C-4′), 129.6 (C-1′), 132.4 (C-4), 135.6 (C-β), 138.8 (C-2), 144.0 (C-5), 152.4 (C-2′), 153.5 (C-5′), 191.4 (C=O). EIMS (70 eV) *m*/*z* (% rel. int.): 330 (40) $[M]^+$.

#### 4.2.14. 2′,3′-Dihydroxy-4′-methoxy-2-(5-methylthienyl)-chalcone (13)

2′,3′-Dihydroxy-4′-methoxyacetophenone (4.6 g, 25 mmol), 3,4-dihydro-α-pyran (13 mL) and pyridinium *p*-toluenesulfonate (0.15 g, 0.6 mmol) were reacted in $CH_2Cl_2$ (100 mL) to give 2′,3′-di(tetra-hydropyran-2-yloxy)-4′- methoxyacetophenone (**13a**). Compound **13a** (8.8 g, 25 mmol), 5-methylthiophene-2-aldehyde (3.2 g, 25 mmol) and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the method described in preparation of **1** to afford **13** (3.48 g, 12.0 mmol, 48%) as red crystals, mp 131–

132 °C. IR (KBr) 3446, 1636, 1558 $cm^{-1}$; $^1H$ NMR ($CDCl_3$): $\delta$ 2.53 (3H, s, $CH_3$), 3.96 (3H, s, $OCH_3$), 5.59 (1H, s, OH), 6.53 (1H, d, $J$ = 9.2 Hz, H-5′), 6.76 (1H, dd, $J$ = 3.6, 1.6 Hz, H-3), 7.18 (1H, d, $J$ = 3.6 Hz, H-4), 7.19 (1H, d, $J$ = 15.6 Hz, H-α), 7.43 (1H, d, $J$ = 9.2 Hz, H-6′), 7.93 (1H, d, $J$ = 15.6 Hz, H-β), 13.30 (1H, s, OH). $^{13}C$ NMR ($CDCl_3$): $\delta$ 15.9 ($CH_3$), 56.2 ($OCH_3$), 102.7 (C-5′), 114.9 (C-1′), 117.4 (C-α), 121.5 (C-3′ and 6′), 127.0 (C-3), 133.5 (C-4), 137.6 (C-β), 138.2 (C-2), 145.3 (C-5), 151.4 (C-4′), 151.9 (C-2′), 192.0 (C=O).[21] EIMS (70 eV) $m/z$ (% rel. int.): 290 (9) $[M]^+$.

#### 4.2.15. 2′,3′,4′-Trihydroxy-2-(5-methylthienyl)chalcone (14)

2′,3′,4′-Trihydroxyacetophenone (4.2 g, 25 mmol), 3,4-dihydro-α-pyran (13 mL) and pyridinium *p*-toluenesulfonate (0.15 g, 0.6 mmol) were reacted in $CH_2Cl_2$ (100 mL) to give 2′,3′,4′-tri(tetra-hydropyran-2-yloxy)acetophenone (**14a**). Compound **14a** (10.5 g, 25 mmol), 5-methylthiophene-2-aldehyde (3.2 g, 25 mmol) and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the method described in preparation of **1** to afford **14** (3.18 g, 11.5 mmol, 46%) as orange crystals, mp 182–183 °C. IR (KBr) 3446, 1624, 1566 $cm^{-1}$; $^1H$ NMR (acetone-$d_6$): $\delta$ 2.54 (3H, s, $CH_3$), 6.51 (1H, d, $J$ = 8.8 Hz, H-5′), 6.88 (1H, dd, $J$ = 3.6, 1.2 Hz, H-3), 7.41 (1H, d, $J$ = 3.6 Hz, H-4), 7.46 (1H, d, $J$ = 15.2 Hz, H-α), 7.60 (1H, d, $J$ = 8.8 Hz, H-6′), 7.83 (1H, s, OH), 7.94 (1H, d, $J$ = 15.2 Hz, H-β), 8.70 (1H, s, OH), 13.56 (1H, s, OH). $^{13}C$ NMR (acetone-$d_6$): $\delta$ 14.6 ($CH_3$), 107.3 (C-5′), 113.4 (C-1′), 117.6 (C-α), 121.9 (C-6′), 122.9 (C-3′), 127.0 (C-3), 133.3 (C-4), 136.6 (C-β), 138.1 (C-2), 145.0 (C-5), 151.7 (C-4′), 153.1 (C-2′), 191.6 (C=O).[21] EIMS (70 eV) $m/z$ (% rel. int.): 276 (14) $[M]^+$.

### 4.3. Tumor cell growth inhibition assays

The cytotoxicity of compounds **2**–**14** against human lung cancer cell A549, human hepatomacellular carcinoma Hep3B, human colorectal adenocarcinoma HT-29 and human breast adenocatcinoma MCF-7 cells obtained from American Type Culture Collection (ATCC, Rockville, MD), were performed by the method as described in the literature.[12]

### 4.4. Flow cytometry

Compounds were added to cells ($1 \times 10^7$). At various time intervals, the reactions were terminated by washing with PBS. The cells were fixed with 4% paraformadehyde/PBS (pH 7.4) at room temperature for 30 min. After certrifugation at 1000 rpm for 10 min, the cells were permeabilized with 0.1% Triton X-100/0.1% sodium citrate at 4 °C for 2 min. Propidium iodide (Sigma) in PBS (10 g/mL) was added to stain the cells at 37 °C for 30 min. The intensity of fluorescence was measured with a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA). A minimum of 5000 cell counts were collected for the analysis by LYSIS II Software.[7]

### 4.5. Mast cell degranulation, neutrophil degranulation, superoxide anion generation, macrophage cultures and drugs treatment and NO determination

The inhibitory assays for chemical mediator induced by various stimulants in mast cells, neutrophils and RAW 264.7 cells were performed by the methods as described in the literature.[22]

### 4.6. Assay of XO activity

The XO activity with xanthine as the substrate was measured at 25 °C, according to the protocol of Kong and others[23] with modification. The assay mixture consisting of 50 μL of test solution, 60 μL of 70 mM phosphate buffer (pH 7.5), and 30 μL of enzyme solution (0.1 unite/mL in 70 mM phosphate buffer (pH 7.5)) was prepared immediately before use. After preincubation at 25 °C for 15 min, the reaction was initiated by addition of 60 μL of substrate solution (150 μM xanthine in the same buffer). The reaction was monitored for 5 min at 295 nm. The XO activity was expressed as micromoles of uric acid per minute.

### 4.7. Statistical analysis

Data were expressed as the mean ± SD. Statistical analysis were performed using the Bonferroni *t*-test method after ANOVA for multigroup comparison and the student's *t*-test method for two group comparison. $P < 0.05$ was considered to be statistically significant.

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## References and notes

1. Mukherjee, S.; Kumar, V.; Prasad, A. K.; Raj, H. G.; Bracke, M. E.; Olsen, C. E.; Jain, S. C.; Parmar, V. S. *Bioorg. Med. Chem.* **2001**, *9*, 337–347.
2. Nielsen, S. F.; Larsen, M. T.; Schønning, B. K.; Kromann, H. *J. Med. Chem.* **2005**, *48*, 2667–2677.
3. Göker, H.; Boykin, D. W.; Yildiz, S. *Bioorg. Med. Chem.* **2005**, *13*, 1707–1714.
4. Bhat, B. A.; Dhar, K. L.; Puri, S. C.; Saxena, A. K.; Shammugravel, M.; Qazi, G. N. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3177–3180.
5. Boeck, P.; Falcão, C. A. B.; Leal, P. C.; Yunes, R. A.; Filho, V. C.; Terres-Santos, E. C.; Rossi-Bergman, B. *Bioorg. Med. Chem.* **2006**, *14*, 1538–1545.
6. Edwards, M. L.; Stemerick, D. M.; Sunkara, P. S. *J. Med. Chem.* **1990**, *33*, 1948–1954.
7. Wei, B. L.; Teng, C. H.; Wang, J. P.; Won, S. J.; Lin, C. N. *Eur. J. Med. Chem.* **2007**, *42*, 660–668.
8. Thiermermann, C.; Van, J. R. *Eur. J. Pharmacol.* **1990**, *182*, 591–595.
9. Honda, T.; Gribble, G. W.; Suh, N.; Finlay, H. J.; Rounds, B. V.; Bore, L.; Flavaloro, F. G., Jr.; Yang, Y.; Sporn, M. B. *J. Med. Chem.* **2000**, *43*, 1866–1877.
10. Robak, J.; Shridi, F.; Wolbis, M.; Krolikowska, M. *Pol. J. Pharmacol. Pharm.* **1988**, *40*, 451–458.
11. Oettl, K.; Reibnebber, G. *Biochim. Biophys. Acta* **1999**, *1430*, 387–395.
12. Won, S. J.; Liu, C. T.; Tsao, L. T.; Weng, J. R.; Ko, H. H.; Wang, J. P.; Lin, C. N. *Eur. J. Med. Chem.* **2005**, *40*, 103–112.
13. Teng, C. H.; Won, S. J.; Lin, C. N. *Bioorg. Med. Chem.* **2005**, *13*, 3439–3445.
14. Hall, M.; Peters, G. *Adv. Cancer Res.* **1996**, *68*, 67–108.
15. Kim, S.; Park, J. H.; Koo, S. Y.; Kim, J. I.; Kim, M. H.; Kim, J. E.; Jo, K. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 6075–6078.
16. Mingghetti, L.; Nicolini, A.; Polazzi, E.; Creminon, C.; Maclouf, J.; Levi, G. *Glia* **1997**, *19*, 152–160.
17. Ding, A. H.; Nathan, C. F.; Stuehr, D. J. *J. Immunol.* **1988**, *141*, 2407–2412.
18. Meda, L.; Cassatella, M. A.; Szendrei, G. I., Jr.; Otves, L. P.; Villalba, B. M.; Ferrari, D.; Ross, F. *Nature* **1995**, *374*, 647–650.
19. Beuther, B.; Cerami, A. *Annu. Rev. Biochem.* **1998**, *57*, 505–518.
20. Rajendran, M.; Manisankar, P.; Gandhidasan, R.; Murugesan, R. *J. Agric. Food Chem.* **2004**, *52*, 7389–7394.
21. Agrawai, P. K. *Carbon-13 NMR of flavonoid*; Elsevier: Amsterdam, 1989. p. 384.
22. Ko, H. H.; Tsao, L. T.; Yu, K. L.; Wang, J. P.; Lin, C. N. *Bioorg. Med. Chem.* **2003**, *11*, 105–111.
23. Kong, L. D.; Zhang, Y.; Pan, X.; Tan, R. X.; Cheng, C. H. *Cell. Mol. Life Sci.* **2000**, *75*, 500–505.