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Structure–Activity Relationship Studies on Chalcone Derivatives: The Potent Inhibition of Chemical Mediators Release

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Abstract—Some chalcones exert potent anti-inflammatory activities. 2′,5′-Dialkoxychalcones and 2′,5′-dihydroxy-4-chloro-dihydrochalcone inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)/interferon-γ (IFN-γ)-activated N9 microglial cells and in LPS-activated RAW 264.7 macrophage-like cells have been demonstrated in our previous reports. These compounds also suppressed the inducible NO synthase (iNOS) expression and cyclooxygenase-2 (COX-2) activity in RAW 264.7 cells. In an effort to continually develop potent anti-inflammatory agent, a series of chalcones were prepared by Claisen–Schmidt condensation of appropriate acetophenones with appropriate aromatic aldehyde and then evaluated their inhibitory effects on the activation of mast cells, neutrophils, macrophages, and microglial cells. Most of the 2′,5′-dihydroxychaclone derivatives exhibited potent inhibitory effects on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with formyl-Met-Leu-Phe (fMLP)/cytochalasin B (CB). Some chalcones showed potent inhibitory effects on superoxide anion generation in rat neutrophils in response to fMLP/CB. Compounds **1** and **5** exhibited potent inhibitory effects on NO production in macrophages and microglial cells. Compound **11** showed inhibitory effect on NO production and iNOS protein expression in RAW 264.7 cells. The present results demonstrated that most of the 2′,5′-dihydroxychaclones have anti-inflammatory effects. The potent inhibitory effect of 2′,5′-dihydroxy-dihydrochaclones on NO production in LPS-activated macrophage, probably through the suppression of iNOS protein expression, is proposed to be useful for the relief of septic shock.

Introduction

It is conceivable that mast cells, neutrophils and macrophages are the important players in inflammatory disorders. Activation of microglial cells also plays a crucial role in inflammatory diseases of the CNS. Thus, inhibition of the activation of these inflammatory cells appears to be an important therapeutic target for small molecule drug design for the treatment of inflammatory diseases. Our recent reports have demonstrated that some synthetic chalcones inhibited the release of chemical mediators from mast cells, neutrophils, macrophages, and microglial cells in vitro, and suppressed the edematous response in vivo.[1–3] We also reported that brousschalcone A, a natural chalcone product isolated from *Broussonetia papyrifera* (Moraceae), exerts a potent antioxidant activity and inhibits the respiratory burst in neutrophils and the inducible nitric oxide synthase (iNOS) expression in macrophages.[4,5] Moreover, a synthetic product, 2′,5′-dihydroxy-4-chloro-dihydrochalcone inhibits the iNOS protein expression and cyclooxygenase-2 (COX-2) activity in RAW 264.7 cells.[6] NO plays a central role in macrophage-induced cytotoxicity and excess NO may contribute to the pathophysiology of septic shock.[7] These findings suggested that some chalcones may be the promising anti-inflammatory agents and have potential in the therapy of septic shock. This report described the chemistry of further synthesized chalcones, biological activity, and the structure–activity relationships of these series of anti-inflammatory agents.

Chemistry

We further prepared a number of new (**1**–**8**) chalcones using Claisen–Schmidt condensation of appropriate

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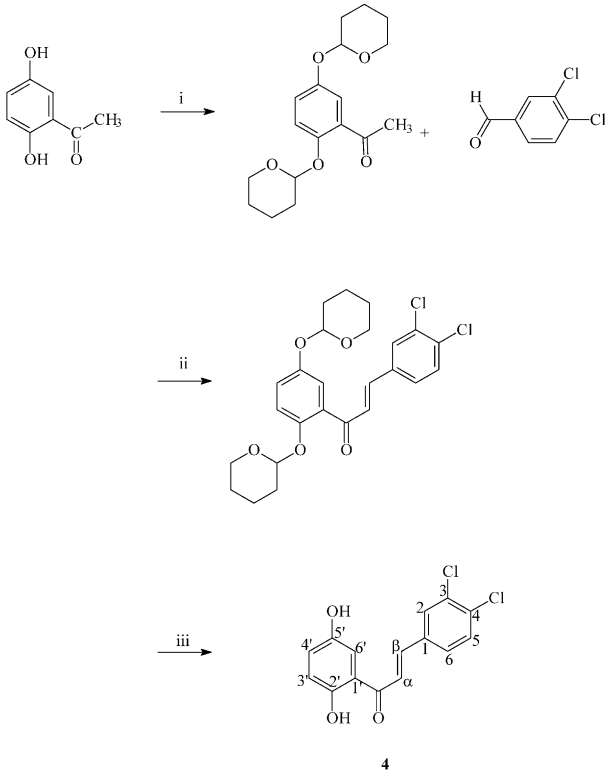
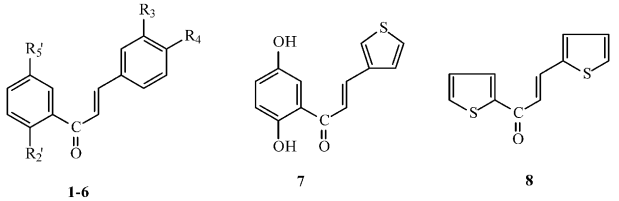
acetophenones or hydroxyacetopheones, protected as tetrahydropyranyl ether, with appropriate aromatic aldehydes or hydroxyaromatic aldehydes, protected as tetrahydropyranyl ether (Scheme 1). This procedure afforded various chalcone derivatives in a good yield (Table 1).



Scheme 1. Reagents: (i) pyridinium *P*-toluenesulfonate, 3,4-dihydro-α-pyran, rt, 4 h; (ii) $BaOH{\cdot}8H_2O$, 40 °C, HCl; (iii) *P*-toluenesulfonic acid, rt, 4 h, 5% $NaHCO_3$.

Table 1. Structure and analytical data of chalcone derivatives



No.	R_2'	R_5'	R_3	R_4	Formula[a]	Mp (°C)	Yield (%)
1	OMe	OMe	H	OH	$C_{17}H_{16}O_4$	127–128[b]	48
2	OMe	OMe	H	Br	$C_{17}H_{15}BrO_3$	85–87[b]	45
3	OMe	OMe	Cl	Cl	$C_{17}H_{14}Cl_2O_3$	121–123[b]	48
4	OH	OH	Cl	Cl	$C_{15}H_{10}Cl_2O_3$	171–174[b]	65
5	OEt	OEt	Cl	Cl	$C_{19}H_{18}Cl_2O_3$	152–153[c]	58
6	OH	OPr	Cl	Cl	$C_{18}H_{16}Cl_2O_3$	97–99[c]	46
7					$C_{13}H_{10}O_3S$	156–157[b]	55
8					$C_{11}H_8OS_2$	95–97[b]	70

[a]C and H analyses were within ±0.4% of the theoretical values.
[b]Crystallizing solvent: $CHCl_3$.
[c]Crystallizing solvent: MeOH.

Biological Results and Discussion

The anti-inflammatory activities of **1**–**8** were studied in vitro for their inhibitory effects on chemical mediators released from mast cells, neutrophils, macrophages, and microglial cells. Compound **4** caused a concentration-dependent inhibition of mast cell degranulation stimulated with compound 48/80 (10 μg/mL) (Table 2). These results indicate that etherifying the 2′,5′-diphenolic or substituting the 2′,5′-dihydroxyphenyl or substituting the B ring of 2′,5′-dihydroxychalcone did not enhance the inhibitory effects on mast cells degranulation, while 2′,5′-dihydroxychalcone with a 3,4-dichlorinated B ring significantly enhanced the inhibitory effects. Mepacrine was used in this experiment as a positive control.

FMLP (1 μM)/CB (5 μg/mL) stimulated the release of β-glucuronidase and lysozyme from rat neutrophils. Compounds **1**–**4**, **6**, and **7** had potent and concentration-dependent inhibitory effects on neutrophil degranulation (Table 3). This clearly indicates that the increase in lipophilicity of **4** or substituting the 3,4-dichlorinated B ring of **4** did not enhance the inhibitory effect. Compounds **5** and **8** had no significant inhibitory

Table 2. The inhibitory effects of chalcone derivatives on the release of β-glucuronidase and histamine from rat peritoneal mast cells stimulated with compound 48/80

Compd	IC_{50} (μM)[a]	
	β-Glucuronidase	Histamine
1	>30 (8.3±2.3)	>30 (10.0±5.1)
2	>30 (28.6±3.4)	>30 (21.7±1.5)
3	>30 (44.3±2.3)	>30 (35.2±1.0)
4	8.9±1.6	11.2±1.8
5	>30 (5.5±4.7)	>30 (12.9±7.9)
6	>30 (49.2±2.0)	>30 (47.6±2.4)
7	>30 (49.1±13.2)	>30 (34.0±11.0)
8	>30 (17.3±2.6)	>30 (20.9±1.2)
Mepacrine	32.2±3.6	48.5±3.8

[a]When 50% inhibition could not be reached at the highest concentration, the% of inhibition is given in parentheses. Data are presented as means ±SEM (n=3–5). Mepacrine was used as a positive control.

Table 3. The inhibitory effects of chalcone derivatives on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with fMLP/CB

Compd	IC_{50} (μM)[a]	
	β-Glucuronidase	Lysozyme
1	20.6±2.0	28.3±1.9
2	5.6±0.3	5.7±0.3
3	5.0±0.3	5.1±0.1
4	1.3±0.1	1.2±0.1
5	>30 (30.6±4.1)	>30 (27.1±5.0)
6	6.2±0.3	6.6±0.4
7	4.6±0.4	6.7±0.3
8	>30 (46.6±0.7)	>30 (30.0±1.4)
Trifluoperazine	18.9±2.1	18.3±0.9

[a]When 50% inhibition could not be reached at the highest concentration, the% of inhibition is given in parentheses. Data are presented as means ±S.E.M. (n=3–5). Trifluoperazine was used as a positive control.

effects in this respect. Hence the enone moiety of chalcones further appears to be required for the inhibition of neutrophils degranulation.[3] Trifluoperazine was used in this study as a positive control.

FMLP/CB or phorbol myristate acetate (PMA) (3 nM) stimulated superoxide anion generation in rat neutrophils. As shown in Table 4, compounds **1**–**4**, **6**, and **8** had potent inhibitory effects on fMLP/CB-induced superoxide anion generation, while **5** and **7** had no significant inhibitory effects. These results indicate that the introduction of a lipophilic alkyl group at the C-2′ and C-5′ positions of **4** might attenuate its inhibitory effects on fMLP/CB-induced responses. In addition, the essential role of the enone moiety of chalcones in the inhibition of fMLP/CB-stimulated superoxide anion generation reconcile our earlier observation.[3] Compound **4** was more potent than the positive control, trifluoperazine. Because fMLP and PMA activate NADPH oxidase to produce superoxide anion through different cellular signaling mechanisms.[8] The observations that compounds **1**–**8** had no appreciable effect on PMA-induced response suggest the involvement of PMA-independent signaling pathway.

Treatment of RAW 264.7 macrophage-like cells with LPS (1 μg/mL) 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 reaction. As shown in Table 5 and Figure 1, LPS induced a significant increase of NO production and this effect was concentration dependently suppressed by **1**, **5**, and 2′,5′-dihydroxydihydrochalcone (**9**), a previously reported compound.[3] The parallel inhibition of NO production in N9 microglial cells as well as in RAW 264.7 cells by **1**–**8** was also observed. The chalcones increased in lipophilicity significantly enhanced the inhibitory effects on NO production. Compounds **9**, and the previously reported compound, 2′,5′-dihydroxy-4-chlorodihydrochalcone (**12**)[6] showed potent inhibitory effects on NO production ($IC_{50} = 11.8 \pm 0.9$ and 4.0 ± 1.6 μM, respectively). Hence 2′,5′-dihydroxy-chalcones may be the potential leading compounds for the development of more potent drugs to inhibition of NO production in macrophages. To determine whether the inhibition of

Table 4. The inhibitory effects of chalcone derivatives on superoxide anion generation in rat neutrophils stimulated with fMLP/CB or PMA

Compd	IC_{50} (μM)[a]	
	fMLP/CB	PMA
1	19.5±3.5	>30 (36.3±6.5)
2	8.0±2.4	>30 (22.9±6.9)
3	12.9±0.4	>30 (−27.1±9.2)
4	0.6±0.2	>10 (21.9±8.7)
5	>30 (32.4±12.2)	>30 (13.2±3.7)
6	17.7±7.2	>30 (49.9±3.4)
7	>1 (43.4±6.1)	>1 (23.5±10.4)
8	24.3±3.2	>30 (35.6±1.6)
Trifluoperazine	12.9±1.0	9.7±1.1

[a]When 50% inhibition could not be reached at the highest concentration, the% of inhibition is given in parentheses. Data are presented as means ±SEM ($n = 3$–5). Trifluoperazine was used as a positive control.

Table 5. The inhibitory effects of chalcone derivatives on the accumulation of NO_2^- in the culture media of RAW 264.7 cells in response to LPS and N9 cells in response to LPS/IFN-γ

Compd	IC_{50} (μM)[a]	
	RAW 264.7 cells	N9 cells
1	14.6±0.1	17.8±0.2
2	>10 (8.9±4.0)	>10 (−5.6±1.1)
3	>3 (2.0±3.1)	>3 (4.3±3.1)
4	>1 (−4.9±1.9)	>1 (−10.3±1.5)
5	4.9±0.3	3.7±0.1
6	>10 (7.5±0.4)	>30 (26.4±2.3)
7	>3 (9.7±2.9)	>3 (10.6±8.2)
8	>30 (12.5±2.2)	>30 (15.2±0.5)
L-NAME[b]	0.51±0.02 mM	0.63±0.01 mM

[a]When 50% inhibition could not be reached at the highest concentration, the% of inhibition is given in parentheses. Data are presented as means ±SEM ($n = 3$–5).
[b]N^{ω}-nitro-L-arginine methyl ether (L-NAME) was used as a positive control.

NO production in RAW 264.7 cells is attributable to the decrease of iNOS protein expression, Western blotting analysis was performed. Unstimulated cells expressed very low level of iNOS protein, whereas LPS (1 μg/mL) induced a large amount of iNOS protein expression (Fig. 2). Compound **9** greatly inhibited the iNOS protein expression, while 2′,5′-dimethoxy-4-chlorochalcone (**10**), a previously reported compound,[3] had little effect. Thus, the blockade of iNOS transcription has a critical role as evidenced from the parallelism of the inhibition of NO production and iNOS protein expression by **9** and **10.**

Conclusions

This study further verifies that most of 2′,5′-dihydroxychalcone derivatives exert potent inhibitory effects on

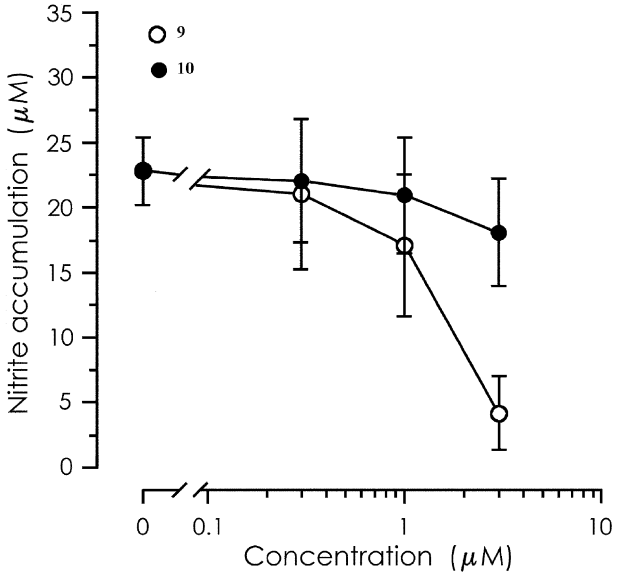


Figure 1. Inhibitory effect of **9** and **10** on nitrite accumulation. RAW 264.7 cells were pretreated with **9** or **10** at indicated concentrations for 1 h before stimulation with 1 μg/mL of LPS for 24 h. The nitrite in the media was measured by the Griess reaction.

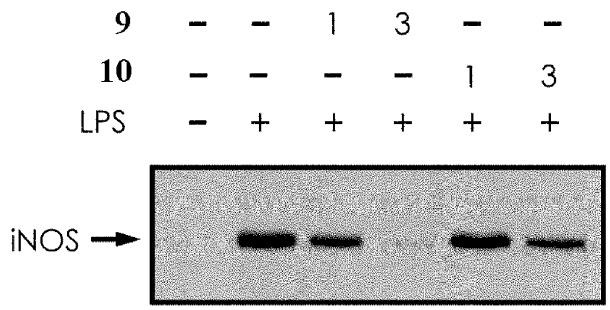


Figure 2. Effects of **9** and **10** on the expression of iNOS protein. RAW 264.7 cells were pretreated with **9** or **10** at 1 or 3 μM for 1 h, followed by stimulation with 1 μg/mL of LPS. After 24 h, the expression of iNOS protein was analyzed by Western blotting. Similar results were obtained from three independent experiments.

the release of chemical mediators from inflammatory cells. NO plays a central role in macrophage-induced cytotoxicity and has been demonstrated to implicate in the pathology of central neurologic diseases and also in the peripheral tissue damage associated with acute and chronic inflammation[9–11] and septic shock.[7] The present study suggests that the inhibition of NO production by **1**, **5**, **9** and **10** in macrophages and **5** in microglial cells may have value in the therapeutic treatment or prevention of certain central as well as peripheral inflammatory diseases associated with the increase of NO production. The inhibitory effects on the COX-2 activity and NO production in RAW 264.7 macrophages are likely to involve a multitude of independent or interrelated mechanisms.

Experimental

Mast cell degranulation

Heparinized Tyrode's solution was injected into the peritoneal cavity of exsanguinated rat (Sprague–Dawley, 250–300 g). After abdominal massage, cells in the peritoneal fluid were harvested and then separated in 38% bovine serum albumin (BSA) in glucose-free Tyrode's solution. Cell pellets were washed and suspended in Tyrode's solution with 0.1% BSA to 1×10^6 cell/mL. Cell suspension was then preincubated at 37°C with DMSO or drugs for 5 min. The final volume of DMSO in the reaction mixture was $\leq$0.5%. Fifteen min after the addition of compound 48/80 (10 μg/mL), β-glucuronidase (phenolphthalein-β-glucuronide as substrate, 550 nm) and histamine (*O*-phthadialdehyde condensation, 350/450 nm) in the supernatant were determined.[12] The total content of β-glucuronidase and histamine was measured after treatment of the cell suspension with Triton X-100, and the percentage release was calculated.

Neutrophil degranulation

Blood was withdrawn from rat and mixed with EDTA. After dextran sedimentation, Ficoll–Hypaque separation and hypotonic lysis of the residual erythrocytes, neutrophils were washed and suspended in Hanks' balanced salt solution (HBSS) to 1×10^7 cell/mL.[13] Cell suspension was preincubated at 37°C with DMSO or drugs for 10 min, and then challenged with fMLP (1 μM)/CB (5 μg/mL). The final volume of DMSO in the reaction mixture was $\leq$0.5%. Forty-five minutes later, the lysozyme (*Micrococcus lysodeikticus* as substrate, 450 nm)[14] and β-glucuronidase in the supernatant were determined. The total content of lysozyme and β-glucuronidase was measured after treatment of the cell suspension with Triton X-100. And the percentage release was calculated.

Superoxide anion generation

Superoxide anion generation was measured in terms of superoxide dismutase-inhibitable cytochrome *c* reduction.[15] Neutrophil suspension was preincubated with DMSO or drugs for 3 min, and then superoxide dismutase or HBSS was added into the blank and test wells, respectively. The final volume of DMSO in the reaction mixture was $\leq$0.5%. After addition of cytochrome *c*, reaction was initiated by challenge with fMLP (0.3 μM)/CB (5 μg/mL) or PMA (3 nM).[9–11] Thirty minutes later, the reaction was terminated by centrifugation and the absorbance changes of supernatant were monitored at 550 nm in a microplate reader.

Macrophage cultures and drugs treatment

RAW 264.7 mouse macrophage-like cell line (American Type Culture Collection) was plated in 96-well tissue-culture plates in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum (FCS), 100 units/mL of penicillin and streptomycin at 2×10^5 cells/200 μL per well. Cells were allowed to adhere overnight. Pretreatment of cells with DMSO or test drugs at 37 °C for 1 h before stimulation with 1 μg/mL of LPS (*Escherichia coli*, serotype 0111:B4) for 24 h, and then the medium was collected and stored at −70 °C until used. The final volume of DMSO in the reaction mixture was $\leq$0.5%.

Microglial cell cultures and drugs treatment

Murine microglial cell lines N9[16] (kindly provided by Dr. P. Ricciardi-Castagnoli, CNR Cellular and Molecular Pharmacology Center, Italy) was plated in 96-well tissue-culture plates in Iscove's Modified Dulbecco's medium containing 5% heat-inactivated FCS and antibiotics at 8×10^4 cells/200 μL per well. Pretreatment of cells with DMSO or test drugs at 37 °C for 1 h before stimulation with LPS (10 ng/mL)/IFN-γ (10 unit/mL) for 24 h, and then the medium was collected and stored at −70 °C until used. The final volume of DMSO in the reaction mixture was $\leq$0.5%.

NO determination

The production of NO was determined in cell medium by measuring the content of nitrite based on the Griess reaction.[17] Briefly, 40 μL of 5 mM sulfanilamide, 10 μL of 2 M HCl, and 20 μL of 40 mM naphthylethylenediamine were sequentially added to 150 μL culture medium.

After 10 min of incubation at room temperature, absorbance was measured at 550 nm in a microplate reader. A standard nitrite curve was generated in the same fashion using $NaNO_2$.

Western blotting analysis

Cells were washed with PBS twice and harvested in Laemmli sodium dodecyl sulfate (SDS) sample buffer. Protein extracts were separated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE), and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature in TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.1% Tween 20) containing 5% nonfat milk. Membranes were washed with TBST buffer and then incubated for 1 h with a monoclonal anti-iNOS antibody (1:1000 dilution). Following washed with TBST buffer, horseradish peroxidase labeled anti-mouse IgG (1:10,000 dilution) was added at room temperature for 1 h. The blots were developed using ECL Western blotting reagents.

Statistical analysis

Data are presented as the means ±S.E.M. Statistical analyses were performed using the Least Significant Difference Test method after analysis of variance. p values < 0.05 were considered to be significant. Analysis of the regression line was used to calculate IC_{50} values.

Chemistry

Melting points (uncorrected) were determined with a Yanaco 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 were obtained on a JMS-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.

Chalcones (**9** and **10**) have been synthesized and reported.[3]

General procedure for obtaining chalcones **1**–**8**.[2]

2′,5′-Dimethoxy-4-hydroxychalcone (1). 4-Hydroxybenzaldehyde (3.05 g, 0.25 mmol) and pyridinum *P*-toluenesulfonate (0.15 g, 0.6 mmol) was stirred for 0.5 h in methylene chloride (80 mL) and then 3,4-dihydro-α-pyran in methylene chloride (13 mL in 20 mL) was added dropwise. The reaction mixture was stirred at room temperature for 4 h, washed twice with water, dried, and evaporated in vacuo. The residue yielded crude 4-(tetrahydropyran-2-yloxy) benzaldehyde (**1a**). A part of crude **1a** was eluted through a silica-gel column with *n*-hexane–CH_2Cl_2 (2:1) to give **1a**: yellowish oil. ^1H NMR ($CDCl_3$): δ 1.37, 1.66 (each 3H, m, –$OCH_2(CH_2)_3CH$=), 3.36 (1H, m, –$OCHCH_2$–), 3.57 (1H, m, –$CHCH_2$–), 5.27 (1H, t, J = 2.8 Hz, –CH_2CH=), 6.90 (2H, dd, J = 8.8, 2.0 Hz, H2 and H-6), 7.56 (2H, dd, J = 8.8, 2.0 Hz, H-3 and H-5), 9.60 (1H, s, –CHO). ^{13}C NMR ($CDCl_3$): δ 17.7 (–$CH_2CH_2CH_2$–), 24.3 (–OCH_2CH_2–), 29.3 (–CH_2CH=), 61.2 (–OCH_2–), 95.4 (–CH_2CH=), 116.0 (C-2 and C-6), 129.7 (C-4), 131.0 (C-3 and C-5), 161.5 (C-1), 190.1 (–CHO). EIMS (70 ev) m/z (% rel. int.): 206 (8) $[M]^+$, 189 (10), 121 (29), 85 (100). HREIMS m/z $[M]^+$ 206.0941 (calcd for $C_{12}H_{14}O_3$, 206.0943). Crude **1a**, 2,5-dimethoxyacetophenone (4.5 g, 25 mmol), and barium hydroxide octahydrate (4.29 g, 25 mmol) were dissolved in MeOH (100 mL). The reaction mixture was stirred for 12 h at 40 °C and then evaporated in vacuo, water (100 mL) was added and the mixture was neutralized with HCl (1 M, 35 mL) and extracted with EtOAc. The organic layer was separated, washed with water, dried, and evaporated in vacuo. The residue yielded crude 2′,5′-dimethoxy-4-(tetrahydropyran-2-yloxy) chalcone (**1b**). A part of crude **1b** was eluted through a silica-gel column with *n*-hexane–CH_2Cl_2 (2:1) to give **1b**: yellowish oil. ^1H NMR ($CDCl_3$): δ 1.56, 1.82 (each 3H, m, –$OCH_2(CH_2)_3CH$=), 3.56 (1H, m, –$OCHCHCH_2$–), 3.72 (3H, s, OMe), 3.78 (3H, s, OMe), 3.83 (1H, m, –$OCHCH_2$–), 5.41 (1H, t, J = 2.8 Hz, –CH_2CH=), 6.86 (1H, d, J = 8.8 Hz, H-3′), 6.91 (1H, dd, J = 8.8, 2.8 Hz, H-4′), 7.02 (2H, dd, J = 8.8, 2.8 Hz, H-3 and H-6), 7.14 (1H, d, J = 2.8 Hz, H-6′), 7.27 (1H, d, J = 15.8 Hz, H-α), 7.48 (2H, dd, J = 8.8, 2.8 Hz, H-2 and -6), 7.58 (1H, d, J = 15.8 Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 18.3 (–$CH_2CH_2CH_2$–), 24.8 (–OCH_2CH_2–), 29.8 (–CH_2CH=), 55.4 (OMe), 56.1 (OMe), 61.7 (–OCH_2–), 95.8 (–CH_2CH=), 113.1 (C-6′), 114.2 (C-3′), 116.4 (C-3 and -5), 118.4 (C-α), 124.6 (C-4′), 128.1 (C-1′), 129.7 (C-2 and -6), 130.1 (C-1), 143.2 (C-β), 152.1 (C-5′), 153.2 (C-2′), 158.7 (C-4), 192.3 (CO). EIMS (70 eV) m/z (% rel. int.): 368 (1) $[M]^+$, 284 (100), 253 (21), 178 (86), 151 (48), 107 (57). HREIMS m/z $[M]^+$ 368.1630 (calcd for $C_{22}H_{24}O_5$, 368.1623). Crude **1b** and *P*-toluenesulphonic acid (0.18 g, 1.05 mmol) were dissolved in MeOH (100 mL). The reaction mixture was stirred for 4 h at room temperature, and then evaporated in vacuo. Water (100 mL) was added to the mixture, neutralized with 5% $NaHCO_3$ (50 mL) and extracted with EtOAc. The organic layer was separated, washed with water, dried, and evaporated in vacuo. The residue was eluted through a silica-gel column with cyclohexane–EtOAc (4:1) to give **1** (3.41 g, 12 mmol, 48%): IR (KBr) 3319, 1650, 1558 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.79 (3H, s, OMe), 3.83 (3H, s, OMe), 6.88 (2H, dd, J = 8.8, 2.0 Hz, H-3 and -5), 6.93 (1H, d, J = 8.8 Hz, H-3′), 7.02 (1H, dd, J = 8.8, 2.8 Hz, H-4′), 7.15 (1H, d, J = 2.8 Hz, H-6′), 7.25 (1H, d, J = 16.0 Hz, H-α), 7.46 (2H, dd, J = 8.8, 2.0 Hz, H-2 and -6), 7.59 (1H, d, J = 16.0 Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 55.8 (OMe), 56.5 (OMe), 113.5 (C-6′), 114.5 (C-3′), 116.1 (C-3 and -5), 118.9 (C-α), 124.3 (C-4′), 127.3 (C-1′), 129.8 (C-1), 130.5 (C-2 and -6), 144.6 (C-β), 152.4 (C-5′), 153.6 (C-2′), 158.6 (C-4), 193.5 (CO). EIMS (70 eV) m/z (% rel. int.): 284 $[M]^+$ (100), 177 (77), 147 (88), 107 (84).

4-Bromo-2′,5′-dimethoxychalcone (2). 2,5-Dimethoxyacetophenone (4.5 g, 25 mmol), 4-bromobenzaldehyde (4.63 g, 25 mmol), and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of **1b** to give **2** (3.91 g, 11.3 mmol, 45%):IR (KBr) 1660, 1600

cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 3.81 (3H, s, OMe), 3.86 (3H, s, OMe), 6.94 (1H, d, J = 9.0 Hz, H-3′), 7.04 (1H, dd, J = 9.0, 3.1 Hz, H-4), 7.19 (1H, d, J = 3.1 Hz, H-6′), 7.41 (1H, d, J = 15.8 Hz, H-α), 7.42–7.52 (4H, m, H-2,-3,-5 and -6), 7.58 (1H, d, J = 15.8 Hz, H-β). ^{13}C NMR ($CDCl_3$) δ 55.8 (OMe), 56.4 (OMe), 113.4 (C-6′), 114.4 (C-3′), 119.4 (C-α), 124.3 (C-1′), 127.3 (C-4′), 129.1 (C-1), 129.7 (C-3,-5), 132.0 (C-2,-6), 134.1 (C-4), 141.5 (C-β), 152.6 (C-5′), 153.6 (C-2′), 191.9 (CO). EIMS (70 eV) m/z (% rel. int.): 348 $[M+1]^+$ (11), 177 (20), 165 (100).

3,4-Dichloro-2′,5′-dimethoxychalcone (3). 2,5-Dimethoxyacetophenone (4.5 g, 25 mmol), 3,4-dichlorobenzaldehyde (4.38 g, 25 mmol), and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of **1b** to give **3** (4.05 g, 12 mmol, 48%): IR (KBr) 1669, 1605 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 3.81 (3H, s, OMe), 3.88 (3H, s, OMe), 6.95 (1H, d, J = 9.2 Hz, H-3′), 7.05 (1H, dd, J = 9.2, 3.2 Hz, H-4′), 7.20 (1H, d, J = 3.2 Hz, H-6′), 7.41 (1H, d, J = 16.0 Hz, H-α), 7.41 (1H, dd, J = 8.4, 2.0 Hz, H-6), 7.47 (1H, d, J = 8.4 Hz, H-5), 7.54 (1H, d, J = 16.0 Hz, H-β), 7.66 (1H, d, J = 2.0 Hz, H-2). ^{13}C NMR ($CDCl_3$): δ 55.9 (OMe), 56.2 (OMe), 113.6 (C-6′), 114.5 (C-3′), 119.8 (C-α), 127.3 (C-4′), 128.4 (C-6), 129.2 (C-1′), 129.8 (C-2), 130.8 (C-5), 133.2 (C-1), 134.0 (C-3), 135.4 (C-4), 139.9 (C-β), 152.8 (C-5′), 153.7 (C-2′), 191.6 (CO). EIMS (70 eV) m/z (%rel. int.): 336 $[M-1]^+$ (11), 165 (100).

3,4-Dichloro-2′,5′-dihydroxychalcone (4). 2,5-Dihydroxyacetophenone (3.8 g, 25 mmol), and pyridinium *P*-toluenesulfonate (0.15 g, 0.6 mmol) were stirred for 0.5 h in methylene chloride (80 mL), and then 3,4-dihydro-α-pyran in methylene chloride (13 mL in 20 mL) was added dropwise. The reaction mixture was stirred at room temperature for 4 h. The reaction mixture was washed twice with water, dried, and evaporated in vacuo. The residue yielded crude 2′,5′-bis(tetrahydropyran-2-yloxy) acetophenone (**4a**). A part of crude **4a** was eluted through a silica-gel column with *n*-hexane–CH_2Cl_2 (2:1) to give **4a**: yellow oil. It was identified with various spectral data and compared with those of authentic sample.[2] Crude **4a**, 3,4-dichlorobenzaldehyde (4.4 g, 25 mmol), and barium hydroxide octahydrate (4.2 g, 25 mmol) were dissolved in MeOH (100 mL). The reaction mixture was stirred for 12 h at 40 °C and then evaporated in vacuo. Water (100 mL) was added and the mixture was neutralized with HCl (1M, 30 mL) and extracted with EtOAc. The organic layer was separated, washed with water, dried, and evaporated in vacuo. The residue yielded crude 2′,5′-bis(tetrahydropyran-2-yloxy)-3,4-dichlorochalcone (**4b**). A part of crude **4b** was eluted through a silica-gel column with *n*-hexane–CH_2Cl_2 (2:1) to give **4b**: yellow powder. ^{1}H NMR: δ 1.65 (6H, m, –OCH(CH_2)$_3$CH=), 1.95 (6H, m, –OCH(CH_2)$_3$CH=), 3.63 (2H, m, –OCH$_2$–), 3.94 (2H, m, –OCH$_2$–), 5.34 (2H, t, J = 2.8 Hz, –CH$_2$CH=×2), 6.94 (1H, d, J = 8.8 Hz, H-3′), 7.09 (1H, d, J = 2.8 Hz, H-6′), 7.29 (1H, dd, J = 8.8, 2.8 Hz, H-4′), 7.44 (1H, d, J = 9.0 Hz, H-3), 7.51 (1H, d, J = 15.8 Hz, H-α), 7.53 (1H, dd, J = 9.0, 3.0 Hz, H-2), 7.53 (1H, d, J = 3.0 Hz, H-6), 7.75 (1H, d, J = 15.8, H-β). ^{13}C NMR ($CDCl_3$): δ 18.7 (–CH$_2$CH_2CH$_2$–), 19.7 (–CH$_2$CH_2CH$_2$–), 25.1 (–OCH$_2$ CH_2–), 25.4 (–OCH$_2$CH_2–), 30.4 (–CH_2CH=), 30.6 (–CH_2CH=), 62.1 (–OCH$_2$–), 62.9 (–OCH$_2$–), 94.6 (–CH$_2$CH=), 97.6 (–CH$_2$CH=), 116.5 (C-3), 119.2 (C-α), 119.4 (C-6′), 121.7 (C-4′), 127.6 (C-6), 130.0 (C-2), 130.9 (C-5), 133.3 (C-1), 134.0 (C-3), 134.7 (C-4), 142.5 (C-β), 149.1 (C-5′), 158.6 (C-2′), 192.7 (CO). EIMS (70 eV) m/z (% rel. int.): 480 (0.02) $[M+4]^+$, 478 (0.1), $[M+2]^+$, 476 (0.2) $[M]^+$, 394 (0.2), 392 (0.3), 310 (43), 308 (70), 136 (100). It failed to show a molecular ion peak under high-resolution conditions. Crude **4b** and *P*-toluenesulfonic acid (0.18 g, 1.05 mmol) were dissolved in MeOH (100 mL). The reaction mixture was stirred for 4 h at room temperature, and then evaporated in vacuo. Water (100 mL) was added to the mixture, neutralized with 5% $NaHCO_3$ (50 mL), and extracted with EtOAc. The organic layer was separated, washed with water, dried, and evaporated in vacuo. The residue was eluted through a silica gel column with cyclohexane–EtOAc (4:1) to give **4** (5.02 g, 16.3 mmol, 65%):IR (KBr) 3434, 1636, 1600 cm^{-1}. ^{1}H NMR ($(CD_3)_2CO$): δ 6.86 (1H, d, J = 8.0 Hz, H-3′), 7.16 (1H, dd, J = 8.0, 2.8 Hz, H-4′), 7.63 (1H, d, J = 2.8 Hz, H-6′), 7.69 (1H, d, J = 8.0 Hz, H-5), 7.85 (1H, d, J = 16.0 Hz, H-α), 7.88 (1H, dd, J = 8.0, 2.8 Hz, H-6), 8.06 (1H, d, J = 16.0 Hz, H-β), 8.16 (1H, bs, OH-5′), 8.19 (1H, d, J = 2.8 Hz, H-2), 12.16 (1H, s, OH-2′). ^{13}C NMR ($(CD_3)_2CO$): δ 116.5 (C-6′), 120.2 (C-α), 121.3 (C-1′), 124.6 (C-3′), 126.9 (C-4′), 130.4 (C-6), 131.8 (C-2), 132.6 (C-5), 134.2 (C-1), 135.3 (C-3), 137.2 (C-4), 143.6 (C-β), 151.0 (C-5′), 158.6 (C-2′), 195.1 (CO). EIMS (70 eV) m/z (% rel. int.): 308 $[M]^+$ (8), 163 (21), 136 (100).

3,4-Dichloro-2′,5′-diethoxychalcone (5). A mixture of (7.7 g, 25 mmol), ethyliodide (8.1 g, 52 mmol), and potassium carbonate (15 g, 25 mmol) in DMF (50 mL) were stirred at room temperature for 18 h. The mixture was diluted with water and washed three times with water. The organic phase was dried over sodium sulfate, filtered, and concentrated in vacuo to give the product. Purification via silica-gel column chromatography yield **5** (5.3 g, 14.5 mmol, 58%): IR (KBr) 1657, 1596 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.40 (6H, m, 2×Me), 4.06 (4H, 2×OCH$_2$), 6.91 (1H, d, J = 9.2 Hz, H-3′), 7.03 (1H, dd, J = 9.2, 3.2 Hz, H-4′), 7.24 (1H, d, J = 3.2 Hz, H-6′), 7.40 (1H, dd, J = 8.4, 2.0 Hz, H-6), 7.46 (1H, d, J = 8.4 Hz, H-5), 7.51 (1H, d, J = 16.0 Hz, H-α), 7.56 (1H, d, J = 16.0 Hz, H-β), 7.66 (1H, d, J = 2.0 Hz, H-2). ^{13}C NMR ($CDCl_3$): δ 14.8 (Me), 15.0 (Me), 64.1 (OCH$_2$), 65.2 (OCH$_2$), 114.7 (C-6′), 115.1 (C-3′), 120.7 (C-α), 127.2 (C-4′), 128.7 (C-6), 129.2 (C-1′), 129.7 (C-2), 130.9 (C-5), 133.2 (C-1), 133.8 (C-3), 135.5 (C-4), 139.2 (C-β), 152.3 (C-5′), 153.1 (C-2′), 191.3 (CO). EIMS (70 eV) m/z (% rel. int.): 366 $[M+1]^+$ (25), 164 (100), 136 (79).

3,4-Dichloro-2′-hydroxy-5′-propoxychalcone (6). A mixture of **4** (7.7 g, 25 mmol), *n*-propyl iodide (8.84 g, 52 mmol), and potassium carbonate (15 g, 25 mmol) in DMF (50 mL) were treated as in synthesis of **5** to give **6** (4.04 g, 11.5 mmol, 46%). IR (KBr): 1646, 1577 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.07 (3H, t, Me), 1.83 (2H, m, CH$_2$), 3.94 (2H, t, OCH$_2$), 6.97 (1H, d, J = 9.0 Hz, H-3′), 7.16 (1H, dd, J = 9.0, 2.9 Hz, H-4′), 7.33 (1H,

$J = 2.9$ Hz, H-6′), 7.46 (1H, dd, $J = 8.3$, 1.7 Hz, H-6), 7.54 (1H, d, $J = 16.0$ Hz, H-α), 7.56 (1H, d, $J = 8.3$ Hz, H-5), 7.74 (1H, d, $J = 1.7$ Hz, H-2), 7.78 (1H, d, $J = 16.0$ Hz, H-β), 12.21 (1H, s, OH). ^{13}C NMR ($CDCl_3$):δ 10.6 (Me), 22.7 (CH_2), 70.7 (OCH_2), 114.0 (C-6′), 119.3 (C-α), 119.5 (C-1′), 121.8 (C-3′), 124.7 (C-4′), 127.7 (C-6), 129.9 (C-5), 131.0 (C-2), 133.4 (C-1), 134.6 (C-3,-4) 142.5 (C-β), 151.3 (C-5′), 157.9 (C-2′), 192.8 (CO). EMIS (70 eV) m/z (% rel. int.): 350 $[M]^+$ (15), 178 (36), 136 (100).

2′,5′-Dihydroxy-3-thienylchalcone (7). Crude **4a**, 3-thiophenaldehyde (2.8 g, 25 mmol), and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of **4** to give **7** (3.4 g, 13.8 mmol, 55%): IR (KBr): 3358, 1642 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 4.92 (1H, s, OH-5′), 6.93 (1H, d, $J = 8.8$ Hz, H-3′), 7.06 (1H, dd, $J = 8.8$, 3.2 Hz, H-4′), 7.35 (1H, d, $J = 3.2$ Hz, H-6′), 7.38 (1H, d, $J = 15.2$ Hz, H-α), 7.39 (1H, dd, $J = 5.2$, 2.8 Hz, H-4), 7.42 (1H, dd, $J = 5.2$, 1.6 Hz, H-5), 7.65 (1H, dd, $J = 2.8$, 1.6 Hz, H-2), 7.90 (1H, d, $J = 15.2$ Hz, H-β), 12.41 (1H, s, OH-2′). ^{13}C NMR ($CDCl_3$): δ 114.5 (C-6′), 119.4 (C-α), 119.7 (C-3′), 119.8 (C-1′), 124.8 (C-4′), 125.5 (C-5), 127.2 (C-2), 130.1 (C-4), 137.9 (C-3), 139.1 (C-β), 147.4 (C-5′), 157.7 (C-2), 193.5 (CO). EMIS (70 eV) m/z (% rel. int.): 246 $[M]^+$ (39), 136 (100).

2′,2-Dithienylchalcone (8). 2-Acetylthiophene (2.8 g, 25 mmol), 2-thiophenaldehyde (2.8 g, 25 mmol), and barium hydroxide octahydrate (4.29 g, 25 mmol), were treated as in the synthesis of **3** to yielded **8** (3.86 g, 17.5 mmol, 70%). IR (KBr): 1638, 1571 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.08 (1H, dd, $J = 5.0$, 3.6 Hz, H-4′), 7.17 (1H, dd, $J = 5.2$, 4.0 Hz, H-4), 7.21 (1H, d, $J = 15.6$ Hz, H-α), 7.36 (1H, d, $J = 5.2$ Hz, H-5), 7.42 (1H, d, $J = 5.0$ Hz, H-5′), 7.66 (1H, dd, $J = 3.6$, 1.2 Hz, H-3′), 7.84 (1H, dd, $J = 4.0$, 0.8 Hz, H-3), 7.96 (1H, d, $J = 15.6$ Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 120.4 (C-α), 128.2 (C-4′), 128.3 (C-3′), 128.8 (C-5′), 131.6 (C-4), 132.1 (C-3), 133.8 (C-5), 136.4 (C-β), 140.1 (C-2), 145.5 (C-2′), 181.5 (CO). EMIS (70 eV) m/z (% rel. int.): 220 $[M]^+$ (100), 191 (68), 111 (57).

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