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ORIGINAL PAPER

Curcumin-like diarylpentanoid analogues as melanogenesis inhibitors

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Abstract Anti-melanogenesis screening of 47 synthesized curcumin-like diarylpentanoid analogues was performed to show that some had a potent inhibitory effect on the melanogenesis in B16 melanoma cells. Their actions were considered to be mostly due to tyrosinase inhibition, tyrosinase expression inhibition, and melanin pigment degradation. The structure–activity relationships of those curcumin-like diarylpentanoid analogues which inhibited the melanogenesis and tyrosinase activity were also discussed. Of those compounds assayed, (2*E*,6*E*)-2,6-bis(2,5-dimethoxybenzylidene)cyclohexanone showed the most potent anti-melanogenesis effect, the mechanism of which is considered to be the degradation of the melanin pigment in B16 melanoma cells, affecting neither the tyrosinase activity nor tyrosinase expression.

Keywords Curcumin-like diarylpentanoids · Anti-melanogenesis · Tyrosinase activity · Tyrosinase expression · Melanin degradation

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Introduction

The essential role of melanins in the skin cells is the absorption of free radicals, and melanogenesis is thus a process of producing photoprotective agents against the damaging effect of ultraviolet [1]. The intracellular pigmentation or melanogenesis can be prevented at various stages in which tyrosinase is a major key enzyme involved [2]. Tyrosinase is a copper-containing enzyme that catalyzes two reactions: the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and the conversion of DOPA to DOPA quinones [3]. These quinones then produce pigment deposition in the skin via enzymatic and nonenzymatic reactions [4]. Thus, for the treatment of skin pigmentations or for the whitening of skin, inhibition of melanin synthesis is essential [5], and tyrosinase inhibitors and antioxidants are often used for the prevention of cellular pigmentation [6].

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a diarylheptanoid isolated from the rhizome of *Curcuma longa* (Zingiberaceae), which is used as a yellow spice or pigment in foods [7]. Besides various biological activities including anti-inflammatory, anticancer, antioxidative, antiangiogenesis, and anti-HIV activities [8–11], curcumin is also known to inhibit tyrosinase activity as do other diarylheptanoids such as yakuchinones A and B [12]. In our previous preliminary paper [13], we reported that some diarylpentanoid analogues inhibited tyrosinase enzyme activity. In the present study, we synthesized 47 diarylpentadiene-3-one curcumin-type analogues(Fig. 1) and evaluated their anti-melanogenesis activity by using B16 melanoma cells. The modes of their actions on tyrosinase activity, tyrosinase protein expression, and/or melanin pigment degradation were also discussed in detail.

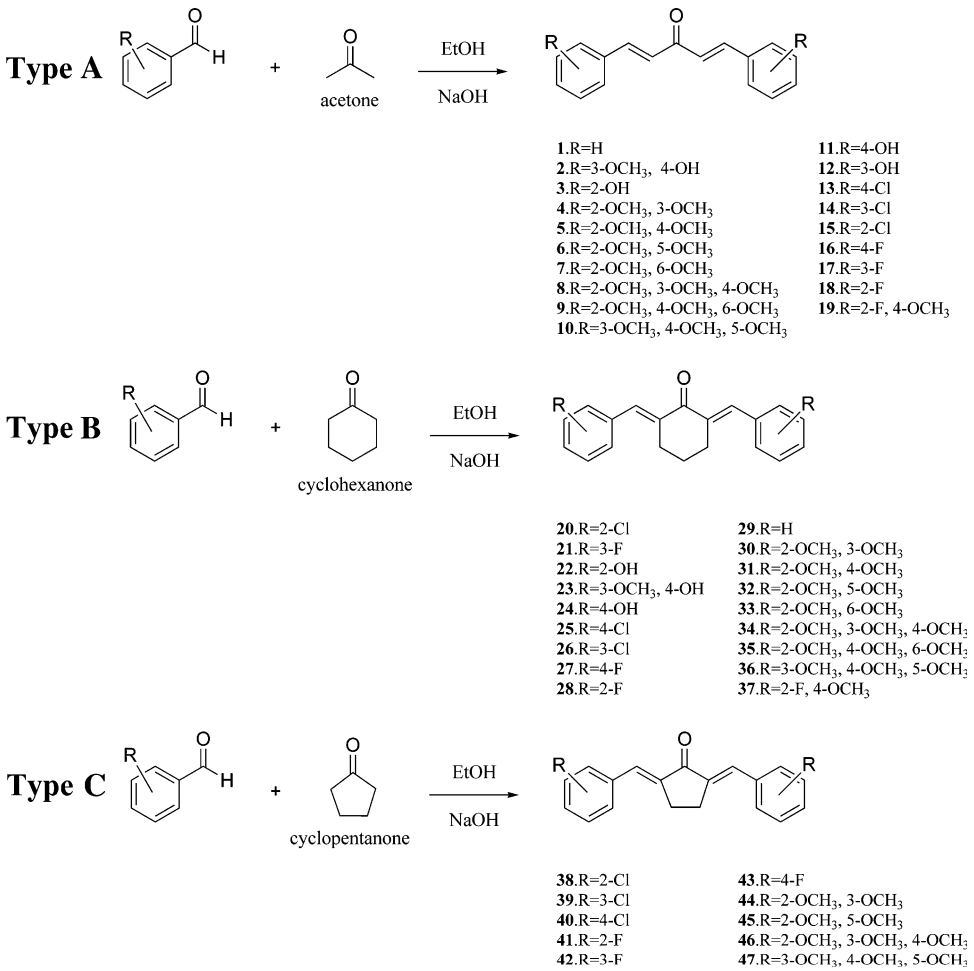

Fig. 1 Synthesis of the diarylpentanoid analogues. The group joining the two aromatic rings is pentadiene-3-one in type A (**1**–**19**), 2,6-dibenzyldienecyclohexanone in type B (**20**–**37**), 2,5-dibenzyldienecyclopentanone in type C (**38**–**47**)

Synthesis of the curcumin analogues

Curcumin analogues **1**–**47** were prepared by the base-catalyzed aldole condensation between an appropriate aromatic aldehyde and ketone in a ratio of 2:1 (Fig. 1). The ketone used for the synthesis of **1**–**19** (type A) was acetone, that for the synthesis of **20**–**37** (type B) was cyclopentanone, and that for the synthesis of **38**–**47** (type C) was cyclohexanone. Compounds **1**–**47** were used in the melanogenesis assay as a dimethyl sulfoxide (DMSO) solution. All the compounds were characterized by the analysis of their spectroscopic data and by the comparison of these data with those of related compounds. Detailed spectroscopic data of the synthesized compounds can be obtained from us on request.

Results and discussion

Effect on melanin production in B16 melanoma cells

The effect on melanin production was assayed by incubating B16 melanoma cells in the presence of compounds **1**–**47** and 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase [14]. Table 1 shows the assay results, or the precentage melanin content in cells along with the viability of the cells treated with the test compounds. Table 1 shows only the results of active analogues. Many of the type-A and -B compounds were shown to be active, whereas those of type C were found to have much less effect on the melanin content in B16 melanoma cells and the cell viability. Compounds **4**, **11**, **20**, **23**, **24**, **30**, **31**, and **32** markedly decreased the melanin contents (<50%) in the range of high cell viability of B16 melanoma cells (>80%) in a dose-dependent manner. Compounds **2**, **6**, **7**, **12**, **28**, **35**, **37**, **38**, **42**, and **44** showed moderate anti-melanogenesis activity. The cyclohexanone-type analogues **30** and **32** (type B) showed more effective anti-melanogenesis activity in comparison with **4** and **6** (type A), and analogues such as **44** and **45** (type C), respectively, in which each of them has the same substituent pattern on the benzene rings. Thus, type-B analogues possessing methoxy groups on the aromatic rings showed the most efficient anti-melanogenesis activity among the 47 synthesized curcumin-like diarylpentanoid analogues.

Effect on tyrosinase activity (anti-tyrosinase activity)

Tyrosinase is a key enzyme in the melanin synthesis in melanogenesis. Tyrosinase catalyzes the hydroxylation of tyrosine to DOPA, the oxidation of DOPA to DOPA quinone, and the oxidation of 5,6-dihydroxyindole (DHI) to indole-quione [3]. Therefore, with the expectation of producing a skin-whitening effect, melanogenesis can be inhibited by the activity of tyrosinase enzyme and/or expression of tyrosinase in cells. Kojic acid, which is known to be a tyrosinase inhibitor [15, 16], has been used in cosmetics as a skin-whitening agent with some success. The anti-tyrosinase effect of curcumin-like diarylpentanoid analogues **1**–**47** on the melanogenesis by tyrosinase was examined by using an in vitro mushroom tyrosinase assay [17, 18]. As shown in Table 1, some type-A and -B compounds inhibited tyrosinase, but scarcely any of type C did. The anti-tyrosinase activities of **2**, **11**, and **12** in this assay were about the same as that of kojic acid, whereas those of **13**, **24**, **35**, and **37** were much weaker. Curcumin itself showed a weak anti-tyrosinase activity (IC_{50} 75.5 μM). Compound **2**, possessing the same substitution pattern on the benzene rings (3-methoxy and 4-hydroxyl) as that of curcumin, showed a higher inhibition effect than curcumin, whereas compound **23** with the same aromatic rings did not show significant inhibitory activity. The result suggests that the acyclic moiety in type-A compounds plays an important role in the anti-tyrosinase activity. Furthermore, the five-carbon length between the two aromatic rings seems to dramatically increase the anti-tyrosinase activity (cf. the results of **2** vs. curcumin), suggesting the primary importance of the structure between the two aromatic rings in the tyrosinase inhibition. As regards the substituents on the aromatic rings, of compounds **3** (no anti-tyrosinase activity), **11**, and **12**, which have a hydroxyl group at the 2-, 4-, and 3-position, respectively, **11** showed potent activity, suggesting the importance of the presence of the 4-hydroxyl group. Of compounds **11**, **13**, and **16** (no anti-tyrosinase

Table 1 Melanin content in B16 melanoma cells after 4 days incubation with test compound and 50% inhibition concentration (IC_{50}, μM) values of anti-tyrosinase activity

Type	Compound	Melanin content in B16 melanoma cells (%)						Anti-tyrosinase
		50 μM	25 μM	12.5 μM	6.3 μM	3.1 μM	1.6 μM	(IC_{50}, μM)
A	**2**	–[c]	–[c]	–[c]	–[c]	63[a]	82[a]	16.3
	4	–[c]	–[c]	–[c]	–[c]	47[a]	76[a]	–[e]
	6	–[c]	–[c]	–[c]	14[a]	56[a]	57[a]	–[e]
	7	–[c]	–[c]	–[c]	17[b]	58[a]	86[a]	–[e]
	11	–[c]	–[c]	17[a]	43[a]	53[a]	83[a]	6.4
	12	–[c]	–[c]	16[a]	62[a]	81[a]	93[a]	12.7
	13	–[c]	–[c]	70[a]	87[a]	–[d]	–[d]	90.4
B	**20**	–[c]	39[a]	70[a]	91[a]	–[d]	–[d]	–[e]
	23	–[c]	–[c]	–[c]	11[a]	42[a]	54[a]	–[e]
	24	–[c]	–[c]	9[a]	51[b]	64[a]	88[a]	43.5
	28	15[a]	65[a]	69[a]	–[d]	–[d]	–[d]	–[e]
	30	–[c]	–[c]	23[a]	68[a]	78[a]	87[a]	–[e]
	31	35[a]	60[a]	70[a]	–[d]	–[d]	–[d]	–[e]
	32	2[a]	17[a]	52[a]	63[a]	74[a]	79[a]	–[e]
	35	7[a]	22[a]	80[a]	–[d]	–[d]	–[d]	98.9
	37	55[a]	88[a]	–[d]	–[d]	–[d]	–[d]	66.1
C	**38**	66[a]	80[a]	–[d]	–[d]	–[d]	–[d]	–[e]
	42	47[a]	–[d]	–[d]	–[d]	–[d]	–[d]	–[e]
	44	55[a]	–[d]	–[d]	–[d]	–[d]	–[d]	–[e]
Curcumin		–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	75.5
Arbutin		73[a]	80[a]	–[d]	–[d]	–[d]	–[d]	–[e]
Kojic acid		–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	12.0

[a] Cell viability greater than 80%

[b] 60–80%

[c] Less than 60%

[d] Not determined

[e] Not active at 100 μM

activity), possessing 4-OH, 4-Cl, and 4-F functions, respectively, only **11** inhibited the tyrosinase activity. The present findings agree with previous results reported [12], implying that the anti-melanogenesis effect exerted by compounds **2**, **11**, and **12** is at least partly due to inhibition of the endogenous tyrosinase enzymatic activity in B16 melanoma cells.

Effect on tyrosinase protein expression in B16 melanoma cells

The cellular tyrosinase protein content in the B16 melanoma cells, assayed by the Western blotting method, showed that type-A (**4** and **11**) and type-B (**20**, **23**, **24**, **30**, **31**, and **32**) compounds had higher anti-melanogenesis activities in the first screening. As shown in Fig. 2, only **4**, **20**, **24**, and **30** decreased the tyrosinase expression in B16 melanoma cells, resulting in reduction of the melanin content in B16 melanoma cells, although **11**, **23**, **31** (data not shown), and **32** (Fig. 3) did not affect the expression levels. Compound **11**, which showed a potent anti-tyrosinase activity, had no effect on the tyrosinase protein content. These results showed that **11** decreased the melanin content in B16 melanoma cells by inhibiting tyrosinase enzyme but not tyrosinase expression in B16 melanoma cells. Compounds **4** and **30**, possessing two methoxy groups at 2- and 3-position on the benzene rings, were able to inhibit the expression of tyrosinase in B16 melanoma cells.

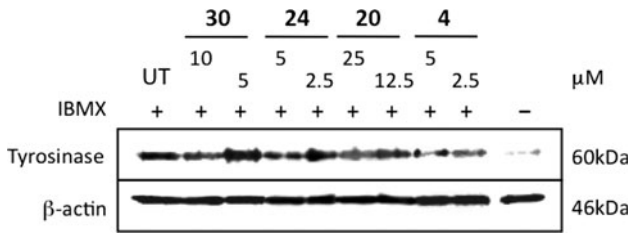

Fig. 2 Effects of compounds **4**, **20**, **24**, and **30** on tyrosinase protein expression in B16 melanoma cells

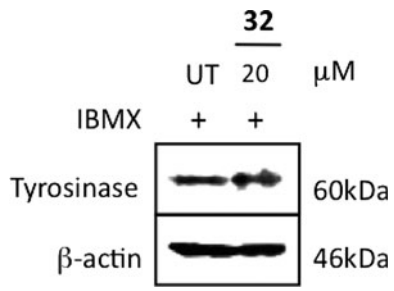

Fig. 3 Effects of compound **32** on tyrosinase protein expression in B16 melanoma cells. **32** did not affect the expression level of tyrosinase in B16 melanoma cells

Compound **32** inhibited neither the tyrosinase activity nor the tyrosinase expression in B16 melanoma cells (Fig. 3). When the cells were treated with IBMX, compound **32** decreased the melanin pigment content considerably, i.e., the whitening effect of **32** on the B16 melanoma cells is apparently tyrosinase-independent (Figs. 4, 5a, b). Almost all compounds reported as having anti-melanogenesis activity have been correlated with tyrosinase enzyme inhibition and/or the expression inhibition, and subsequently effective whitening. However, the mechanism of **32** might be different from these cases. Thus, the mechanism of the whitening effect by **32** is unique, and considered to be due to the degradation of melanin pigment in B16 melanoma cells, which suggests that compound **32** is a good candidate as a skin-whitening agent.

Conclusion

Forty-seven curcumin-like analogues of three types (A–C), each with a different group linking the two aromatic rings, were assayed for their anti-melanogenesis activity by using B16 melanoma cells. The first screening of anti-melanogenesis in B16 melanoma cells indicated that compounds of type A and B had a more potent anti-melanogenesis effect than those of type C. Of those compounds, compound **11** exhibited the most potent anti-tyrosinase activity, although it showed scarcely any effect on the tyrosinase expression in B16 melanoma cells. Of those compounds assayed, compound **32** was shown to be the most desirable skin-whitening agent. The mechanism was considered to be the degradation of the melanin pigment in B16 melanoma cells, affecting neither the tyrosinase activity nor tyrosinase expression. Compound **32** might be a promising cosmetic skin-whitening agent.

Experimental

Chemistry

All chemicals and reagents are of research grade and purchased from either Sigma-Aldrich, Merck, or Acros Organics. All solvents used in experiment were either HPLC-grade or dried and distilled prior to use. The typical

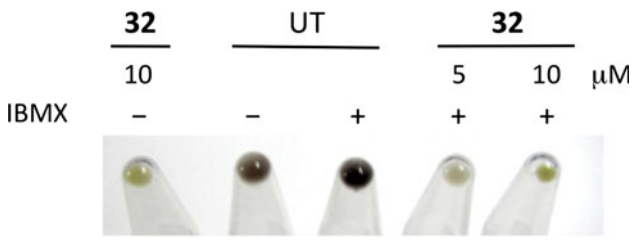

Fig. 4 B16 melanoma cells treated with **32** with or without IBMX

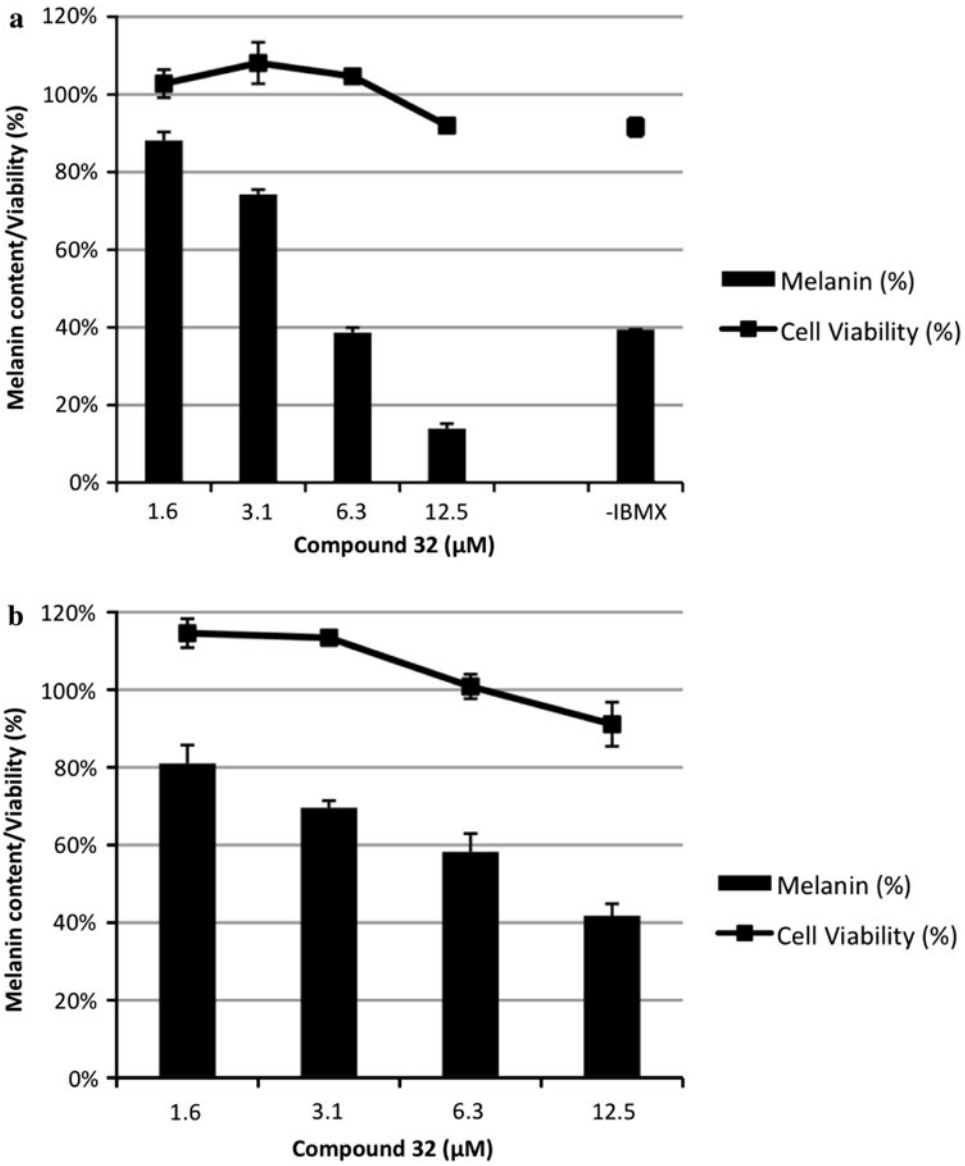

Fig. 5 Melanin content in B16 melanoma cells treated with **32** with (**a**) or without IBMX (**b**)

workup included washing with brine and drying the organic layer with magnesium sulfate (anhydrous) before concentration in vacuo. Analytical TLC was carried out on silica gel F_{254} precoated (0.2 mm thickness, Merck) aluminum TLC sheets. The TLC plates were spotted with samples using a fine glass capillary tube and developed in a chromatographic tank saturated with solvent vapor at room temperature. Melting points were determined using a hot stage melting point apparatus equipped with a microscope, XSP-12 model 500X, and are uncorrected. IR spectra were recorded on a Perkin Elmer RXI FT-IR spectrometer as KBr discs or thin films. ^{1}H NMR spectra were recorded on a Varian Unity 500 spectrometer (Varian Inc., Palo Alto, CA, USA) and measured at 500 MHz. Deuterated chloroform ($CDCl_3$) and acetone-d_6 were used as solvents. Chemical shifts (δ) are reported in ppm. Mass spectra (MS) were recorded on a Polaris Q ThermoFinnigan (San Jose, CA, USA) with ionization-induced by electron impacts (EIMS) at 70 eV.

General procedure for synthesis of analogues (1–47)

The bis-aldol adducts were prepared the dropwise addition of aqueous NaOH (20 wt%, 15 mL, 75 mmol) into a vigorously stirred solution of the appropriate benzaldehyde (51 mmol) and ketone (25 mmol) in absolute EtOH (20 mL). The reaction was stirred at room temperature for 48 h, and 100 mL distilled water was then added. The resulting solution was neutralized by gently bubbling CO_2 gas through it and the yellow precipitate produced was filtered off, washed with distilled water, and dried under vacuum. The products were purified by recrystallization from an appropriate solvent, typically a MeOH–water mixture.

*(1E,4E)-1,5-Diphenylpenta-1,4-dien-3-one (**1**)*

Mp 112–114°C; UV (MeOH): λ_{max} 326 (ε 28,700), 326 (15,500), 202 (37,600) nm; IR (KBr): ν_{max} 1,574, 1,593, 1,651 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.83 (m, 5H), 7.50 (m, 5H), 7.35 (d, 2H, $J = 16.5$ Hz); MS (EI): *m/z* 234 $[M]^+$.

*(1E,4E)-1,5-Bis(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**2**)*

Mp 82–83°C; UV (MeOH): λ_{max} 388 (ε 28,900), 260 (12,700), 203 (31,200) nm; IR (KBr): ν_{max} 1,513, 1,639, 3,398 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.70 (d, 2H, $J = 16.0$ Hz), 7.40 (d, 2H, $J = 1.5$ Hz), 7.24 (dd, 2H, $J = 8.5$, 1.5 Hz), 7.14 (d, 2H, $J = 16.0$ Hz), 6.91 (d, 2H, $J = 8.5$ Hz), 3.95 (s, 6H); MS (EI): *m/z* 326 $[M]^+$.

*(1E,4E)-1,5-Bis(2-hydroxyphenyl)penta-1,4-dien-3-one (**3**)*

Mp 156°C; UV (MeOH): λ_{max} 371 (ε 22,600), 312 (15,400), 256 (8,700), 211 (31,500) nm; IR (KBr): ν_{max} 1,555, 1,604, 3,207 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 8.09 (d, 2H, $J = 16.0$ Hz), 7.72 (dd, 2H, $J = 8.0$, 1.5 Hz), 7.34 (d, 2H, $J = 16.0$ Hz), 7.27 (td, 2H, $J = 8.5$, 1.5 Hz), 6.99 (d, 2H, $J = 8.5$ Hz), 6.93 (t, 2H, $J = 7.5$ Hz); MS (EI): *m/z* 248 $[M-H_2O]^+$.

*(1E,4E)-1,5-Bis(2,3-dimethoxyphenyl)penta-1,4-dien-3-one (**4**)*

Mp 106–108°C; UV (MeOH): λ_{max} 333 (ε 36,000), 204 (60,700) nm; IR (KBr): ν_{max} 1,577, 1,619, 1,737 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 8.07 (d, 2H, $J = 16.0$ Hz), 7.41 (t, 2H, $J = 4.5$ Hz), 7.31 (d, 2H, $J = 16.0$ Hz), 7.14 (d, 4H, $J = 4.5$ Hz), 3.91 (s, 6H), 3.89 (s, 6H); MS (EI): *m/z* 354 $[M]^+$.

*(1E,4E)-1,5-Bis(2,4-dimethoxyphenyl)penta-1,4-dien-3-one (**5**)*

Mp 138–140°C; UV (MeOH): λ_{max} 392 (ε 30,400), 251 (16,200), 301 (50,100) nm; IR (KBr): ν_{max} 1,504, 1,588, 1,645 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 8.00 (d, 2H, $J = 15.5$ Hz), 7.71 (d, 2H, $J = 8.5$ Hz), 7.17 (d, 2H, $J = 15.5$ Hz), 6.66 (d, 2H, $J = 2.0$ Hz), 6.62 (dd, 2H, $J = 8.5$, 2.0 Hz), 3.96 (s, 6H), 3.89 (s, 6H); MS (EI): *m/z* 354 $[M]^+$.

*(1E,4E)-1,5-Bis(2,5-dimethoxyphenyl)penta-1,4-dien-3-one (**6**)*

Mp 105–106°C; UV (MeOH): λ_{max} 396 (ε 20,000), 316 (21,000), 207 (38,300) nm; IR (KBr): ν_{max} 1,493, 1,578, 1,611 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.98 (d, 2H, $J = 15.5$ Hz), 6.88 (d, 2H, $J = 15.5$ Hz), 6.84 (d, 4H, $J = 8.0$ Hz), 6.82 (s, 2H), 3.96 (s, 6H), 3.85 (s, 6H); MS (EI): *m/z* 354 $[M]^+$.

*(1E,4E)-1,5-Bis(2,6-dimethoxyphenyl)penta-1,4-dien-3-one (**7**)*

Mp 153–155°C; UV (MeOH): λ_{max} 366 (ε 28,100), 213 (42,900) nm; IR (KBr): ν_{max} 1,568, 1,604, 1,637 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 8.17 (d, 2H, $J = 16.3$ Hz), 7.59 (d, 2H, $J = 16.3$ Hz), 7.36 (t, 2H, $J = 8.5$ Hz), 6.75 (d, 4H, $J = 8.5$ Hz), 3.90 (s, 12H); MS (EI): *m/z* 354 $[M]^+$.

*(1E,4E)-1,5-Bis(2,3,4-trimethoxyphenyl)penta-1,4-dien-3-one (**8**)*

Mp 89–91°C; UV (MeOH): λ_{max} 366 (ε 15,200), 203 (43,800) nm; IR (KBr):ν_{max} 1,496, 1,590, 1,611 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.89 (d, 2H, $J = 16.0$ Hz), 7.47 (d, 2H, $J = 16.0$ Hz), 7.20 (d, 2H, $J = 8.5$ Hz), 6.88 (d, 2H, $J = 8.5$ Hz), 3.91 (s, 6H), 3.87 (s, 6H), 3.84 (s, 6H); MS (EI): *m/z* 414 $[M]^+$.

*(1E,4E)-1,5-Bis(2,4,6-trimethoxyphenyl)penta-1,4-dien-3-one (**9**)*

Mp 209–211°C; UV (MeOH): λ_{max} 384 (ε 10,400), 254 (7,100), 201 (32,100) nm; IR (KBr): ν_{max} 1,561, 1,602, 1,629 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 8.13 (d, 2H, $J = 16.0$ Hz), 7.43 (d, 2H, $J = 16.0$ Hz), 6.33 (s, 4H), 3.97 (s, 12H), 3.90 (s, 6H); MS (EI): *m/z* 414 $[M]^+$.

*(1E,4E)-1,5-Bis(3,4,5-trimethoxyphenyl)penta-1,4-dien-3-one (**10**)*

Mp 105–107°C; UV (MeOH): λ_{max} 366 (ε 23,200), 200 (38,100) nm; IR (KBr): ν_{max} 1,504, 1,582, 1,620 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) 7.71 (d, 2H, $J = 16.0$ Hz), 7.24 (d, 2H, $J = 16.0$ Hz), 7.09 (s, 4H), 3.92 (s, 12H), 3.80 (s, 6H); MS (EI): *m/z* 414 $[M]^+$.

*(1E,4E)-1,5-Bis(4-hydroxyphenyl)penta-1,4-dien-3-one (**11**)*

Mp 244–246°C; UV (MeOH): λ_{max} 375 (ε 34,200), 242 (16,400), 201 (28,500) nm; IR (KBr): ν_{max} 1,504, 1,582, 1,620, 3,321 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.71 (d, 2H, $J = 16.0$ Hz), 7.63 (d, 4H, $J = 8:0$ Hz), 7.10 (d, 2H, $J = 16.0$ Hz), 6.93 (d, 4H, $J = 8.0$ Hz); MS (EI): *m/z* 266 $[M]^+$.

*(1E,4E)-1,5-Bis(3-hydroxyphenyl)penta-1,4-dien-3-one (**12**)*

Mp 198–200°C; UV (MeOH): λ_{max} 329 (ε 27,300), 254 (111,200), 212 (39,300) nm; IR (KBr): ν_{max} 1,581, 1,620, 3,387 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.70 (d, 2H, $J = 16.0$ Hz), 7.29 (t, 2H, $J = 7.6$ Hz), 7.22 (d, 2H, $J = 16.0$ Hz), 7.21 (d, 2H, $J = 7.6$ Hz), 7.11 (s, 2H), 6.93 (dd, 2H, $J = 8$, 2.4 Hz); MS (EI): *m/z* 266 $[M]^+$.

*(1E,4E)-1,5-Bis(4-chlorophenyl)penta-1,4-dien-3-one (**13**)*

Mp 184–186°C; UV (MeOH): λ_{max} 322 (ε 26,800), 225 (20,100), 201 (32,300) nm; IR (KBr): ν_{max} 1,091, 1,491, 1,589, 1,651 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.80 (d, 2H, $J = 15.9$ Hz), 7.52 (d, 4H, $J = 8.5$ Hz), 7.37 (d, 4H, $J = 8.5$ Hz), 7.34 (d, 2H, $J = 15.9$ Hz); MS (EI): *m/z* 303 $[M]^+$.

*(1E,4E)-1,5-Bis(3-chlorophenyl)penta-1,4-dien-3-one (**14**)*

Mp 123–126°C; UV (MeOH): λ_{max} 316 (ε 22,300), 202 (45,600) nm; IR (KBr): ν_{max} 1,077, 1,594, 1,655 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.83 (s, 2H), 7.78 (d, 2H, $J = 15.9$ Hz), 7.73 (d, 2H, $J = 7.0$ Hz), 7.54 (m, 4H), 7.42 (d, 2H, $J = 15.9$ Hz); MS (EI): *m/z* 303 $[M]^+$.

*(1E,4E)-1,5-Bis(2-chlorophenyl)penta-1,4-dien-3-one (**15**)*

Mp 114–116°C; UV (MeOH): λ_{max} 321 (ε 21,600), 203 (40,500) nm; IR (KBr): ν_{max} 1,099, 1,587, 1,618 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 8.11 (d, 2H, $J = 16.1$ Hz), 7.97 (dd, 2H, $J = 7.5$, 1.5 Hz), 7.56 (dd, 2H, $J = 7.5$, 1.5 Hz), 7.47 (m, 4H), 7.34 (d, 2H, $J = 16.1$ Hz); MS (EI): *m/z* 303 $[M]^+$.

*(1E,4E)-1,5-Bis(4-fluorophenyl)penta-1,4-dien-3-one (**16**)*

Mp 90–92°C; UV (MeOH): λ_{max} 324 (ε 23,000), 225 (15,900), 201 (28,800) nm; IR (KBr): ν_{max} 1,237, 1,508, 1,634 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.86 (d, 2H, $J = 15.9$ Hz), 7.52 (d, 4H, $J = 8.5$ Hz), 7.27 (d, 4H, $J = 8.5$ Hz), 7.17 (d, 2H, $J = 15.9$ Hz); MS (EI): *m/z* 270 $[M]^+$.

*(1E,4E)-1,5-Bis(3-fluorophenyl)penta-1,4-dien-3-one (**17**)*

Mp 132–134°C; UV (MeOH): λ_{max} 316 (ε 23,900), 201 (38,900) nm; IR (KBr): ν_{max} 1,248, 1,581, 1,594, 1,655 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.83 (d, 2H, $J = 15.5$ Hz), 7.59 (m, 6H), 7.38 (d, 2H, $J = 15.5$ Hz), 7.23 (m, 2H); MS (EI): *m/z* 270 $[M]^+$.

*(1E,4E)-1,5-Bis(2-fluorophenyl)penta-1,4-dien-3-one (**18**)*

Mp 68–70°C; UV (MeOH): λ_{max} 312 (ε 24,100), 201 (37,300) nm; IR (KBr): ν_{max} 1,230, 1,486, 1,626 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.83 (d, 2H, $J = 16.0$ Hz), 7.63 (dd, 4H, $J = 7.5$ Hz, 2.0 Hz), 7.57 (m, 2H), 7.38 (d, 2H, $J = 16.0$ Hz), 7.26 (td, 2H, $J = 7.5$, 2.0 Hz); MS (EI): *m/z* 270 $[M]^+$.

*(1E,4E)-1,5-Bis(2-fluoro-4-methoxyphenyl)penta-1,4-dien-3-one (**19**)*

Mp 115–117°C; UV (MeOH): λ_{max} 357 (ε 33,900), 325 (17,600), 200 (37,800) nm; IR (KBr): ν_{max} 1,105, 1,508, 1,612 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.84 (d, 2H, $J = 15.5$ Hz), 7.81 (m, 2H), 7.24 (d, 2H, $J = 15.5$ Hz), 6.90 (m, 4H), 3.91 (s, 6H); MS (EI): *m/z* 330 $[M]^+$.

*(2E,6E)-2,6-Bis(2-chlorobenzylidene)cyclohexanone (**20**)*

Mp 90–92°C; UV (MeOH): λ_{max} 313 (ε 21,700), 203 (46,000) nm; IR (KBr): ν_{max} 1,051, 1,576, 1,603 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.85 (s, 2H), 7.56 (m, 4H), 7.45 (m, 4H), 2.87 (t, 4H, $J = 6.5$ Hz), 1.78 (q, 2H, $J = 6.5$ Hz); MS (EI): *m/z* 343 $[M]^+$.

*(2E,6E)-2,6-Bis(3-fluorobenzylidene)cyclohexanone (**21**)*

Mp 73–75°C; UV (MeOH): λ_{max} 323 (ε 29,500), 229 (16,200), 201 (39,000) nm; IR (KBr): ν_{max} 1,170, 1,582, 1,612 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.67 (s, 2H), 7.54 (m, 2H), 7.39 (d, 2H, $J = 8.0$ Hz), 7.33 (d, 2H, $J = 10.0$ Hz), 7.20 (m, 2H), 3.00 (t, 4H, $J = 6.5$ Hz), 1.84 (q, 2H, $J = 6.5$ Hz); MS (EI): *m/z* 310 $[M]^+$.

*(2E,6E)-2,6-Bis(2-hydroxybenzylidene)cyclohexanone (**22**)*

Mp 146–148°C; UV (MeOH): λ_{max} 356 (ε 19,200), 257 (13,700), 206 (47,900) nm; IR (KBr): ν_{max} 1,550, 1,604, 3,365 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.98 (s, 2H), 7.39 (d, 2H, $J = 8.0$ Hz), 7.24 (t, 2H, $J = 8.0$ Hz), 6.97 (d, 2H, $J = 7.5$ Hz), 6.93 (t, 2H, $J = 7.5$ Hz), 2.92 (t, 4H, $J = 5.5$ Hz), 1.75 (quintet, 2H, $J = 5.5$ Hz); MS (EI): *m/z* 288 $[M-H_2O]^+$.

*(2E,6E)-2,6-Bis(4-hydroxy-3-methoxybenzylidene)cyclohexanone (**23**)*

Mp 187–189°C; UV (MeOH): λ_{max} 383 (ε 37,900), 253 (17,300), 201 (54,500) nm; IR (KBr): ν_{max} 1,513, 1,577, 3,375 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm:

7.65 (s, 2H), 7.16 (s, 2H), 7.10 (d, 2H, $J = 8.2$ Hz), 6.93 (d, 2H, $J = 8.2$ Hz), 3.92 (s, 6H), 2.98 (t, 4 H, $J = 5.5$ Hz), 1.81 (q, 2H, $J = 5.5$ Hz); MS (EI): *m/z* 366 $[M]^+$.

*(2E,6E)-2,6-Bis(4-hydroxybenzylidene)cyclohexanone (**24**)*

Mp 171–173°C; UV (MeOH): λ_{max} 369 (ε 35,400), 243 (18,700), 200 (36,700) nm; IR (KBr): ν_{max} 1,510, 1,594, 3,348 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.65 (s, 2H), 7.45 (d, 4H, $J = 8.5$ Hz), 6.93 (d, 2H, $J = 8.5$ Hz), 2.93 (t, 4 H, $J = 6.0$ Hz), 1.79 (quintet, 2H, $J = 6.0$ Hz); MS (EI): *m/z* 306 $[M]^+$.

*(2E,6E)-2,6-Bis(4-chlorobenzylidene)cyclohexanone (**25**)*

UV (MeOH): λ_{max} 331 (ε 40,300), 235 (22,200), 201 (40,300) nm; IR (KBr): ν_{max} 1,092, 1,489, 1,576, 1,606 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) 7.67 (s, 2H), 7.57 (d, 4H, $J = 8.5$ Hz), 7.49 (d, 4H, $J = 8.5$ Hz), 2.98 (t, 4H, $J = 6.0$ Hz), 1.83 (quintet, 2H, $J = 6.0$ Hz); MS (EI): *m/z* 343 $[M]^+$.

*(2E,6E)-2,6-Bis(3-chlorobenzylidene)cyclohexanone (**26**)*

Mp 136–138°C; UV (MeOH): λ_{max} 323 (ε 38,100), 234 (21,400), 207 (58,400) nm; IR (KBr): ν_{max} 1,092, 1,577, 1,606 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.65 (s, 2H), 7.56 (s, 2H), 7.51 (t, 4H, $J = 5.5$ Hz), 7.44 (m, 2H), 2.99 (m, 4H), 1.86 (quintet, 2H, $J = 6.0$ Hz); MS (EI): *m/z* 343 $[M]^+$.

*(2E,6E)-2,6-Bis(4-fluorobenzylidene)cyclohexanone (**27**)*

Mp 86–88°C; UV (MeOH): λ_{max} 328 (ε 35,400), 230 (21,000), 201 (36,600) nm; IR (KBr): ν_{max} 1,158, 1,507, 1,608 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.68 (s, 2H), 7.56 (s, 2H), 7.49 (d, 2H, $J = 8.3$ Hz), 7.42 (d, 4H, $J = 8.3$ Hz), 2.99 (t, 4H, $J = 5.5$ Hz), 1.83 (quintet, 2H, $J = 6.0$ Hz); MS (EI): *m/z* 310 $[M]^+$.

*(2E,6E)-2,6-Bis(2-fluorobenzylidene)cyclohexanone (**28**)*

Mp 137–139°C; UV (MeOH): λ_{max} 320 (ε 28,200), 227 (15,500), 201 (38,100) nm; IR (KBr): ν_{max} 1,167, 1,483, 1,614 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.78 (s, 2H), 7.58 (t, 2H, $J = 8.0$ Hz), 7.49 (m, 2H), 7.29 (m, 4H), 2.91 (t, 4H, $J = 6.0$ Hz), 1.81 (quintet, 2H, $J = 6.0$ Hz); MS (EI): *m/z* 310 $[M]^+$.

*(2E,6E)-2,6-Dibenzylidenecyclohexanone (**29**)*

Mp 92–94°C; UV (MeOH): λ_{max} 328 (ε 33,800), 231 (16,600), 201 (42,300) nm; IR (KBr): ν_{max} 1,575, 1,607, 1,661 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.72 (s, 2H), 7.56 (d, 4H, $J = 8.0$ Hz), 7.49 (t, 4H, $J = 8.0$ Hz), 7.39 (dd, 2H, $J = 1.5$ Hz, 8.0 Hz), 2.99 (m, 4H), 1.83 (quintet, 2H, $J = 6.0$ Hz); MS (EI): *m/z* 274 $[M]^+$.

*(2E,6E)-2,6-Bis(2,3-dimethoxybenzylidene)cyclohexanone (**30**)*

Mp 107–109°C; UV (MeOH): λ_{max} 326 (ε 31,000), 202 (58,400) nm; IR (KBr): ν_{max} 1,573, 1,591, 1,609 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.90 (s, 2H), 7.12 (m, 4H), 7.03 (dd, 2H, $J = 1.5$ Hz, 7.5 Hz), 3.90 (s, 6H), 3.81 (s, 6H), 2.88 (m, 4H), 1.76 (quintet, 2H, $J = 6.0$ Hz); MS (EI): *m/z* 394 $[M]^+$.

*(2E,6E)-2,6-Bis(2,4-dimethoxybenzylidene)cyclohexanone (**31**)*

Mp 110–112°C; UV (MeOH): λ_{max} 373 (ε 3,700), 247 (3,300), 200 (21,000) nm; IR (KBr): ν_{max} 1,501, 1,573, 1,599 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.92 (s, 2H), 7.38 (d, 2H, $J = 8.5$ Hz), 6.64 (d, 2H, $J = 1.5$ Hz), 6.64 (dd, 2H, $J = 8.$ 5 Hz, 1.5 Hz) 3.89 (s, 6H), 3.87 (s, 6H), 2.91 (t, 4 H, $J = 6.0$ Hz), 1.77 (quintet, 2H, $J = 6.0$ Hz); MS (EI): *m/z* 394 $[M]^+$.

*(2E,6E)-2,6-Bis(2,5-dimethoxybenzylidene)cyclohexanone (**32**)*

Mp 161–163°C; UV (MeOH): λ_{max} 376 (ε 8,700), 313 (11,000), 279 (9,400), 202 (30,100) nm; IR (KBr): ν_{max} 1,494, 1,582, 1,603 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.89 (s, 2H), 7.02 (d, 2H, $J = 8.5$ Hz), 6.97 (d, 4H, $J = 8.5$ Hz), 3.84 (s, 6H), 3.80 (s, 6H), 2.92 (t, 4H, $J = 6.0$ Hz), 1.78 (quintet, 2H, $J = 6.0$ Hz); MS (EI): *m/z* 394 $[M]^+$.

*(2E,6E)-2,6-Bis(2,6-dimethoxybenzylidene)cyclohexanone (**33**)*

Mp 128–130°C; UV (MeOH): λ_{max} 328 (ε 19,700), 202 (67,600) nm; IR (KBr): ν_{max} 1,583, 1,616, 1,669 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.91 (s, 2H), 7.36 (t, 2H, $J = 8.5$ Hz), 6.75 (d, 4H, $J = 8.5$ Hz), 3.87 (s, 6H), 3.81 (s, 6H), 2.90 (t, 4H, $J = 6.0$ Hz), 1.77 (quintet, 2H, $J = 6.0$ Hz); MS (EI): *m/z* 394 $[M]^+$.

*(2E,6E)-2,6-Bis(2,3,4-trimethoxybenzylidene)cyclohexanone (**34**)*

Mp 163–165°C; UV (MeOH): λ_{max} 359 (ε 37,800), 202 (68,700) nm; IR (KBr): ν_{max} 1,493, 1,573, 1,596 cm^{-1}; ^1H

NMR (500 MHz, acetone-d_6) δ in ppm: 7.89 (s, 2H), 7.20 (d, 2H, $J = 8.5$ Hz), 6.88 (d, 2H, $J = 8.5$ Hz), 3.91 (s, 6H), 3.87 (s, 6H), 3.84 (s, 6H), 2.90 (t, 4H, $J = 6.5$ Hz), 1.77 (quintet, 2H, $J = 6.5$ Hz); MS (EI): *m/z* 454 $[M]^+$.

*(2E,6E)-2,6-Bis(2,4,6-trimethoxybenzylidene)cyclohexanone (**35**)*

Mp 197–199°C; UV (MeOH): λ_{max} 353 (ε 8,900), 201 (45,300) nm; IR (KBr): ν_{max} 1,494, 1,584, 1,606 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.90 (s, 2H), 6.86 (s, 4H), 3.97 (s, 12H), 3.90 (s, 6H), 2.89 (t, 4H, $J = 6.0$ Hz), 1.78 (quintet, 2H, $J = 6.0$ Hz); MS (EI): *m/z* 454 $[M]^+$.

*(2E,6E)-2,6-Bis(3,4,5-trimethoxybenzylidene)cyclohexanone (**36**)*

Mp 147–149°C; UV (MeOH): λ_{max} 357 (ε 18,300), 547 (10,400), 200 (42,900) nm; IR (KBr): ν_{max} 1,505, 1,604, 1,579 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.64 (s, 2H), 6.86 (s, 4H), 3.90 (s, 12H), 3.79 (s, 6H), 2.82 (t, 4H, $J = 6.5$ Hz), 1.77 (quintet, 2H, $J = 6.5$ Hz); MS (EI): *m/z* 454 $[M]^+$.

*(2E,6E)-2,6-Bis(2-fluoro-4-methoxybenzylidene)cyclohexanone (**37**)*

Mp 165–167°C; UV (MeOH): λ_{max} 350 (ε 27,600), 231 (22,300), 201 (43,300) nm; IR (KBr): ν_{max} 1,163, 1,504, 1,617 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.76 (s, 2H), 7.54 (t, 2H, $J = 8.5$ Hz), 6.85 (m, 4H), 3.89 (s, 6H), 2.91 (t, 4H, $J = 6.5$ Hz), 1.82 (t, 2H, $J = 6.5$ Hz); MS (EI): *m/z* 370 $[M]^+$.

*(2E,5E)-2,5-Bis(2-chlorobenzylidene)cyclopentanone (**38**)*

Mp 215–217°C; UV (MeOH): λ_{max} 340 (ε 41,900), 235 (17,100), 204 (57,600) nm; IR (KBr): ν_{max} 1,039, 1,601, 1,621 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.84 (s, 2H), 7.80 (d, 2H, $J = 7.5$ Hz), 7.59 (d, 2H, $J = 7.5$ Hz), 7.47 (m, 4H), 3.1 (s, 4H); MS (EI): *m/z* 328 $[M]^+$.

*(2E,5E)-2,5-Bis(3-chlorobenzylidene)cyclopentanone (**39**)*

Mp 216–218°C; UV (MeOH): λ_{max} 340 (ε 25,800), 233 (10,800), 202 (36,500) nm; IR (KBr): ν_{max} 1,099, 1,606, 1,625, 1,690 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.73(s, 2H), 7.69 (d, 2H, $J = 8.0$ Hz), 7.56 (t, 2H, $J = 8.0$ Hz), 7.48 (d, 4H, $J = 12.0$ Hz), 3.22 (s, 4H); MS (EI): *m/z* 328 $[M]^+$.

*(2E,5E)-2,5-Bis(4-chlorobenzylidene)cyclopentanone (**40**)*

Mp 197–199°C; UV (MeOH): λ_{max} 357 (ε 7,700), 235 (4,700), 200 (19,600) nm; IR (KBr): ν_{max} 1,092, 1,585, 1,608, 1,623, 1,695 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.47 (s, 2H), 7.74 (d, 4H, $J = 8.0$ Hz), 7.55 (d, 4H, $J = 8.0$ Hz), 3.19 (s, 4H); MS (EI): *m/z* 328 $[M]^+$.

*(2E,5E)-2,5-Bis(2-fluorobenzylidene)cyclopentanone (**41**)*

Mp 180–182°C; UV (MeOH): λ_{max} 340 (ε 22,100), 228 (12,700), 201 (29,500) nm; IR (KBr): ν_{max} 1,214, 1,603, 1,625, 1,694 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.82 (t, 2H, $J = 8.0$ Hz), 7.71 (s, 2H), 7.52 (m, 2H), 7.36 (t, 2H, $J = 8.0$ Hz), 7.27 (t, 2H, $J = 10$ Hz), 3.17 (s, 4H); MS (EI): *m/z* 296 $[M]^+$.

*(2E,5E)-2,5-Bis(3-fluorobenzylidene)cyclopentanone (**42**)*

Mp 215–217°C; UV (MeOH): λ_{max} 345 (ε 37,100), 230 (15,200), 201 (38,300) nm; IR (KBr): ν_{max} 1,212, 1,578, 1,601, 1,626 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.57 (s, 2H), 7.54 (d, 2H, $J = 8.5$ Hz), 7.48 (d, 4H, $J = 8.5$ Hz), 7.24 (m, 2H), 3.22 (s, 4H); MS (EI): *m/z* 296 $[M]^+$.

*(2E,5E)-2,5-Bis(4-fluorobenzylidene)cyclopentanone (**43**)*

Mp 135–137°C; UV (MeOH): λ_{max} 353 (ε 31,100), 230 (14,000), 200 (31,700) nm; IR (KBr): ν_{max} 1,231, 1,508, 1,586, 1,595 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.80 (dd, 4H, $J = 4.5$ Hz, 8.5 Hz), 7.48 (s, 2H), 7.28 (t, 4H, $J = 8.5$ Hz), 3.17 (s, 4H); MS (EI): *m/z* 296 $[M]^+$.

*(2E,5E)-2,5-Bis(2,3-dimethoxybenzylidene)cyclopentanone (**44**)*

Mp 155–157°C; UV (MeOH): λ_{max} 356 (ε 37,800), 213 (52,400) nm; IR (KBr): ν_{max} 1,574, 1,602, 1,616 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.87 (s, 2H), 7.29 (d, 2H, $J = 8.0$ Hz), 7.19 (m, 4H), 3.91 (s, 6H), 3.86 (s, 6H), 3.09 (s, 4H); MS (EI): *m/z* 380 $[M]^+$.

*(2E,5E)-2,5-Bis(2,5-dimethoxybenzylidene)cyclopentanone (**45**)*

Mp 169–171°C; UV (MeOH): λ_{max} 410 (ε 25,300), 329 (20,300), 281 (11,200), 208 (46,400) nm; IR (KBr): ν_{max} 1,495, 1,585, 1,615 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.89 (s, 2H), 7.20 (d, 2H, $J = 2.5$ Hz), 7.05 (d, 2H, $J = 9$ Hz), 7.01 (d, 2H, $J = 2.5$ Hz), 3.88 (s, 6H), 3.83 (s, 6H), 3.14 (s, 4H); MS (EI): *m/z* 380 $[M]^+$.

*(2E,5E)-2,5-Bis(2,3,4-trimethoxybenzylidene)cyclopentanone (**46**)*

Mp 147–149°C; UV (MeOH): λ_{max} 394 (ε 40,400), 248 (14,300), 210 (44,600) nm; IR (KBr): ν_{max} 1,495, 1,586, 1,615 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.79 (s, 2H), 7.44 (d, 2H, $J = 8.5$ Hz), 6.94 (d, 2H, $J = 8.5$ Hz), 3.93 (s, 12H), 3.85 (s, 6H), 3.07 (s, 4H); MS (EI): *m/z* 440 $[M]^+$.

*(2E,5E)-2,5-Bis(3,4,5-trimethoxybenzylidene)cyclopentanone (**47**)*

Mp 195–197°C; UV (MeOH): λ_{max} 386 (ε 24,600), 246 (7,800), 214 (30,000) nm; IR (KBr): ν_{max} 1,505, 1,578, 1,622 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ in ppm: 7.42 (s, 2H), 7.01 (s, 4H), 3.93 (s, 12H), 3.81 (s, 6H), 3.22 (s, 4H); MS (EI): *m/z* 440 $[M]^+$.

Biological activity

Materials

Murine melanoma cells (B16) were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan. The culture medium used was Dulbecco's modified Eagle medium (DMEM, Sigma), supplemented with fetal bovine serum (10% FBS, Biowest), penicillin (100 U/mL), and streptomycin (0.1 mg/mL, Wako). Other reagents, 3-isobutyl-1-methylxanthine (IBMX, Sigma), arbutin (Sigma), L-(−)-tyrosine (TCI), mushroom tyrosinase (Sigma-Aldrich), disodium hydrogen phosphate, (Wako), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma), were purchased from the respective suppliers.

Measurement of melanin content

B16 melanoma cells precultured in DMEM supplemented with 10% FBS and penicillin/streptomycin (5,000 cells in 100 μL) were added to each well of a 96-well microtiter plate, and were preincubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. Then the cells were cultured in the fresh DMEM containing IBMX (100 μM) with DMSO (control) or the DMSO solution of test compounds of different concentrations (100 μM) for 48 h. Then the medium was replaced by a fresh medium containing the same concentration of compound, and the cells were incubated for 48 h. The medium was removed and the cells were dissolved in 50 μL of 1 N NaOH, which was heated at 90°C for 1 h. The relative melanin content was estimated spectrophotometrically by the absorbance at 400 nm [19]. Arbutin was used as a positive control.

Cell viability assay

The cell viability was determined by the MTT assay [20] after the cultivation. MTT solution (5 mg/mL, 15 μL) was added to each well. After a further 2-h incubation, the medium was removed, and DMSO (50 μL) was added to each well to dissolve the formazan crystals formed. The optical density was measured on a microplate reader equipped with a two-wavelength system (550 and 700 nm). In each experiment, three replicates were prepared for each sample. The percentage of living cells was determined on the basis of the differences in the absorbance between those with the test samples and the controls.

Anti-tyrosinase assay

Tyrosinase activity was spectrophotometrically determined as in the case of the L-tyrosine oxidase activity assay of mushroom tyrosinase [17, 18], with some modifications. Briefly, 0.1 M phosphate buffer (pH 6.8, 500 μL) with or without test samples, and 2.5 mM L-tyrosine (300 μL) were mixed in an Eppendorf tube. Part of the mixtures (200 μL) was transferred to four wells of a 96-well microtiter plate. The absorbance at 475 nm was read on the microplate reader (blank value). Then, 200 units/mL tyrosinase (50 μL) was added to each well. After incubation (37°C, 15 min), the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically by reading the absorbance at 475 nm on a microplate reader. The tyrosinase activity of each test sample was calculated as follows: % inhibition $= 100 - [(A - B)/C] \times 100$ where A is the OD_{475} of a mixture of enzyme and test sample; B the OD_{475} of test sample, but without enzyme; C the OD_{475} with enzyme, but without test sample. Kojic acid was used as a positive control.

Western blotting

Cells treated with test compounds were lysed in lysis buffer, and the extract was passed through a 7.5% SDS–PAGE. The protein fraction was transferred to an Immun-Blot poly(vinylidene difluoride) membrane (PVDF, Bio-Rad) and blocked for 1 h with 5% skim milk. The blotted membrane was incubated with primary antibodies (diluted 1:2,000) for anti-tyrosinase (Santa Cruz) overnight, or 1:2,000 for β-actin (Sigma) for 1 h, followed by horseradish peroxide-conjugated rabbit or mouse IgG (Amersham). Protein bands were visualized with ECL solution (GE Healthcare).

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References

1. Yasui H, Sakurai H (2003) Age-dependent generation of reactive oxygen species in the skin of live hairless rats exposed to UVA light. Exp Dermatol 12:655–661
2. Kim YJ, Uyama H (2005) Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. Cell Mol Life Sci 62:1707–1723
3. Hearing V, Jiménz M (1987) Mammalian tyrosinase. The critical regulatory control point in melanocyte pigmentation. Int J Biochem 19:1141–1147
4. Francois R, Luisa KA, Zalfa AAM, Vincent JH (2005) MC1R and the response of melanocytes to ultraviolet radiation. Mutat Res 571:133–152
5. Funasaka Y, Komoto M, Ichihashi M (2000) Depigmenting effect of α-tocopheryl ferulate on normal human melanocytes. Pigment Cell Res 13:170–174
6. Sturm RA, Teasdale RD, Box NF (2001) Human pigmentation genes: identification, structure and consequences of polymorphic variation. Gene 277:49–62
7. Srinivasan KR (1953) A chromatographic study of the curcuminoids in *Curcuma longa* L. J Pharm Pharmacol 5:448–457
8. Srimal RC, Dhawan BN (1973) Pharmacology of diferuloyl methane (curcumin), a non-steroidal anti-inflammatory agent. J Pharm Pharmacol 25:447–452
9. Chandru H, Sharada AC, Bettadaiah BK, Kumar CS, Rangappa KS, Sunila JK (2007) In vivo growth inhibitory and anti-angiogenic effects of synthetic novel dienone cyclopropoxy curcumin analogs on mouse Ehrlich ascites tumor. Bioorg Med Chem 15:7696–7703
10. Weber WM, Hunsaker LA, Abcouwer SF, Deck LM, Vander Jagt DL (2005) Anti-oxidant activities of curcumin and related enones. Bioorg Med Chem 13:3811–3820
11. Jordan WC, Drew CR (1996) Curcumin—a natural herb with anti-HIV activity. J Nat Med Assoc 88:333
12. Shirota S, Miyazaki K, Aiyama R, Ichioka M, Yokokura T (1994) Tyrosinase inhibitors from crude drugs. Biol Pharm Bull 17:266–269
13. Lee KH, Aziz FHA, Syahida A, Abas F, Shaari K, Israf DA, Lajis NH (2009) Synthesis and biological evaluation of curcumin-like diarylpentanoid analogues for anti-inflammatory, antioxidant and anti-tyrosinase activities. Eur J Med Chem 44:3195–3200
14. Beavo JA, Rogers NL, Crofford OB, Hardman JG, Sutherland EW, Newman EV (1970) Effects of xanthine derivatives on lipolysis and on adenosine 3′,5′-monophosphate phosphodiesterase activity. Mol Pharmacol 6:597–603
15. Chen JS, Wei CI, Marshall MR (1991) Inhibition mechanism of kojic acid on polyphenol oxidase. J Agric Food Chem 11:1897–1901
16. Kahn V (1995) Effect of kojic acid on the oxidation of DL-DOPA, norepinephrine, and dopamine by mushroom tyrosinase. Pigment Cell Res 8:234–240
17. Lerch K (1987) Monophenol monooxygenase from *Neurospora crassa*. Methods Enzymol 142:165–169
18. Matsuda H, Nakashima S, Oda Y, Nakamura S, Yoshikawa M (2009) Melanogenesis inhibitors from the rhizomes of *Alpinia officinarum* in B16 melanoma cells. Bioorg Med Chem 17:6048–6053
19. Tsuboi T, Kondoh H, Hiratsuka J, Mishima Y (1998) Enhanced melanogenesis induced by tyrosinase gene-transfer increases boron-uptake and killing effect of boron neutron capture therapy for amelanotic melanoma. Pigment Cell Res 11:275–282
20. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63