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Structural requirement of chalcones for the inhibitory activity of interleukin-5

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Abstract—Novel chalcones were found as potent inhibitors of interleukin (IL)-5. 1-(2-Benzyloxy-6-hydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one (**2b**, 78.8% inhibition at 50 μM, IC_{50} = 25.3 μM) was initially identified as a potent inhibitor of IL-5. This shows the compatible activity with budesonide or sophoricoside. To identify structural requirements, 26 chalcones were prepared and their inhibitory activities were tested against IL-5. Among them, compound 4-[(*E*)-3-(2-cyclohexylmethoxy-6-hydroxyphenyl)-3-oxoprop-1-enyl]benzenesulfonamide (**2w**, 99.5% inhibition at 50 μM, IC_{50} = 1.8 μM) shows the most potent activity. The important structural requirements of these chalcone analogs exhibiting the inhibitory activity against IL-5 were recognized as the following. (1) The hydrophobic group such as benzyloxy or cyclohexylmethoxy at 6-position of A ring is necessary. (2) The existence of phenolic hydroxyl at 6-position of A ring is critical. (3) Propenone unit as α,β-unsaturated ketone is essential. (4) Electron withdrawing groups with hydrogen acceptor property at 4-position of B ring enhance the activity and quantitative structure–activity relationship of **2** regarding these substituents was determined.

1. Introduction

Eosinophilic inflammation the main histological correlate of airway hyperresponsiveness and tissue injury in the pathogenesis of bronchial asthma.[1,2] There is strong evidence for a central role of T helper type 2 (Th2) cytokines that contributes importantly to diseases such as asthma, and therapeutic strategies that target the Th2 cytokines are of potential benefit in allergic disease.[3,4] Interleukin (IL)-5 appears to be one of the main proinflammatory mediators among a growing number of cytokines and chemokines that induce eosinophilic inflammation.[5,6] IL-5 displays its cellular response by binding to a specific receptor (IL-5R), composed of two distinct polypeptides of α and β chains.[7] The IL-5Rα by itself binds to IL-5 with low affinity.[8] Even though IL-5Rβ alone does not bind to IL-5, it is required for high affinity binding in combination with IL-5Rα and is essential for signal transduction.[9] IL-5Rβ is shared by IL-3 and GM-CSF receptors as the common signal transducer, and thereby these cytokines display several overlapping biological effects.[10,11] Rather than coordinating T-cell effector development or antibody isotype secretion by B-cells, biological effects of IL-5 are confined primarily to growth, differentiation, survival, chemotaxis, and activation of eosinophils.[12–14]. The contribution of IL-5 to allergic disease has been greatly demonstrated by the generation of mice deficient in the IL-5 gene. In contrast to normal mice, allergic IL-5-deficient mice do not generate eosinophilia in the bone marrow, blood or lung in response to allergen provocation.[15] Airway instillation of recombinant IL-5 to allergic IL-5-deficient mice completely restores allergen-induced eosinophilia to levels normally

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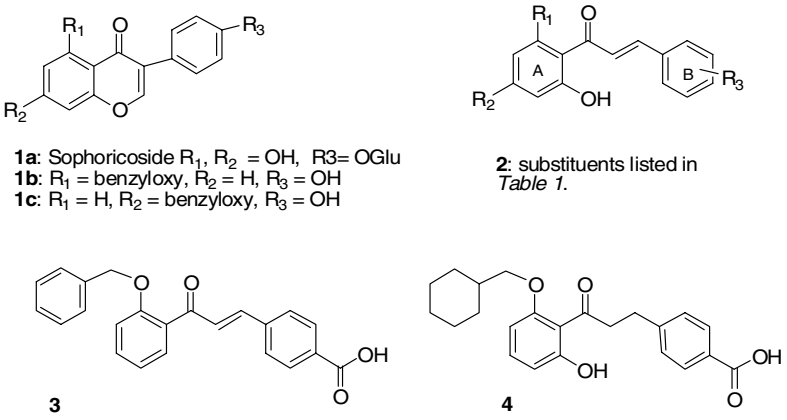


Figure 1. Isoflavonoids (**1**) and chalcone analogs.

observed in allergic wild-type mice.[16] Thus, IL-5 is critically involved in eosinophilia-associated allergic inflammation.

Interfering with the action of IL-5 represents one of the new immunomodulatory therapeutic strategies in the treatment of allergic diseases including bronchial asthma. Compared to established immunosuppressive agents like corticosteroids, a major advantage of this strategy is the specificity of reducing eosinophilic inflammation, thus possibly acting nearly without side effects. However small organic compounds to inhibit IL-5 activity have been rarely found. The isothiazolones were identified as the IL-5 antagonists through modification of Cys66 in IL-5Rα.[17] Sophoricoside (**1a**) and its analogs (Fig. 1) were isolated from *Sophora japonica*, a plant of Leguminosae family, as inhibitors of interleukin (IL)-5 bioactivity,[18] and showed differential inhibition on IL-3 and GM-CSF bioactivities.[19] During the study of structure–activity relationship of sophoricoside

Table 1. Inhibitory activity of chalcone analogs against IL-5

Entry	Compound	Substituents			% inhibition at 50 μM[a]	IC_{50}[a] (μM)
		R_1	R_2	R_3 (σ_p)		
1	**2a**	H	H	4-OH	50.0 ± 2.2	48.2
2	**2b**	BNO[b]	H	4-OH	78.8 ± 1.4	25.3
3	**2c**[20]	BNO	H	4-OCH_2OCH_3	47.8 ± 4.6	>50
4	**2d**	H	BNO	4-OH	77.6 ± 1.4	28.8
5	**2e**	CHMO[c]	H	4-OH (−0.37)	99.0 ± 0.5	12.6
6	**2f**	CHMO	H	4-OCH_2OCH_3	13.0 ± 2.7	>50
7	**2g**	H	CHMO	4-OH	59.7 ± 1.8	21.4
8	**2h**	BNO	H	4-COOH	100.0 ± 0.0	10.2
9	**2i**	H	BNO	4-COOH	100.0 ± 0.0	14.2
10	**2j**	CHMO	H	4-COOH (0)[f]	100.0 ± 0.0	9.4
11	**2k**	CHMO	H	4-$COOCH_3$ (0.45)	98.6 ± 0.1	3.9
12	**2l**	H	CHMO	4-COOH	100.0 ± 0.0	10.8
13	**2m**	CHMO	H	3-COOH	98.1 ± 1.1	14.1
14	**2n**	CHMO	H	2-COOH	−18.9 ± 0.1	>50
15	**2o**	H	H	4-COOH	−18.7 ± 0.1	>50
16	**2p**	CHMO	H	H (0)	99.6 ± 1.7	14.3
17	**2q**	CHMO	H	Cl (0.06)	98.8 ± 0.0	7.1
18	**2r**	CHMO	H	4-NH_2	16.4 ± 11.8	>50
19	**2s**	CHMO	H	4-$NHCOCH_3$ (0)	99.2 ± 0.1	6.4
20	**2t**	CHMO	H	4-CH_2CH_3 (−0.15)	82.3 ± 0.6	27.9
21	**2u**	CHMO	H	4-CH_2OH (0)	99.3 ± 0.0	6.4
22	**2v**	CHMO	H	4-CHO (0.42)	99.8 ± 0.1	4.5
23	**2w**	CHMO	H	4-SO_2NH_2 (0.57)	99.5 ± 0.0	1.8
24	**2x**	BNO	H	4-SO_2NH_2	99.5 ± 0.0	7.0
25	**3**[d]	BNO	H	4-COOH	15.0 ± 1.0	>50
26	**4**[e]	CHMO	H	4-COOH	15.6 ± 0.1	>50
29	Budesonide				70.3 ± 2.1	26.8

[a] Data are means ± SD obtained from triplicate experiments.
[b] BNO, benzyloxy.
[c] CHMO, cyclohexylmethoxy.
[d] Compound **3**; 4-[(*E*)-3-(2-benzyloxyphenyl)-3-oxoprop-1-enyl]benzoic acid.
[e] Compound **4**; 4-[3-(2-cyclohexylmethoxyphenyl)-3-oxoprpyl]benzoic acid.
[f] σ_p value (0) for carboxylate anion was used for finding equation 1 as a real form of carboxylic acid under the bioassay condition.

analogs,[20] chalcones **2a**–**2d** had been prepared as intermediates for the preparation of the corresponding isoflavonones and their inhibitory effects on IL-5 bioactivity have been evaluated as shown in Table 1. The inhibitory activity of **2b** (78.8% inhibition at 50 μM, IC_{50} = 25.3 μM) and **2d** (77.6% inhibition at 50 μM, IC_{50} = 28.8 μM) are compatible with the most potent 5-benzyloxy-3-(4-hydroxyphenyl)chromen-4-one (**1b**, 87.9% inhibition at 50 μM, IC_{50} = 15.3 μM).[20] Although a number of chalcones had been investigated for the various biological properties such as anticancer,[21–23] enzyme inhibitors,[24,25] inhibitor of IL-1 biosynthesis,[26] and antimalarials,[27] chalcones have never been investigated as an inhibitor of IL-5. Thus a number of chalcone derivatives **2**, **3** and its saturated analog **4** were prepared and their inhibitory activities were tested against IL-5 bioactivity for establishing the structure–activity relationship.

2. Chemistry

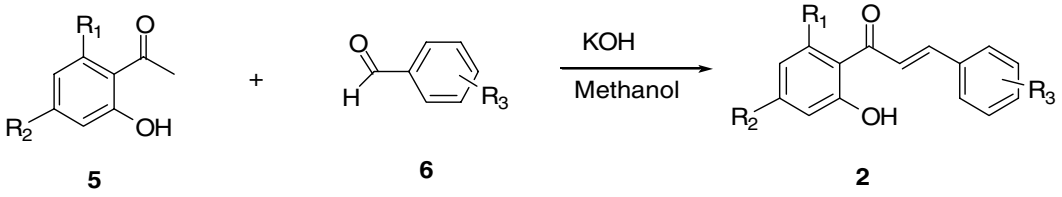
Preparation of **2** was accomplished by the aldol condensation of substituted 2-hydroxyacetophenones **5**[20] and appropriate benzaldehydes **6** in the presence of sodium hydroxide or potassium hydroxide in ethanol at 50 °C for 2 h as illustrated in Scheme 1.[20] The substituents of **2** are listed in Table 1. *Trans* stereochemistry of propenone moiety of **2** was confirmed by the coupling constants of vinyl hydrogens (15–16 Hz). Acetophenones **5** used in the preparation of **2** were prepared by the partial alkylation of 2,6 (or 4)-dihydroxyacetophenone.[20] Most benzaldehydes **6** used in preparation of **2** are commercially available. 4-Formylbenzenesulfonamide used for the preparation of **2w** and **2x** was synthesized by van Es's procedure.[28] Compound **2k** was prepared by esterification of **2j**. Compound **2s** was converted to **2r** by the hydrolysis. Chalcone **3** was obtained by the reaction of 2-benzyloxyacetophenone and 4-formylbenzoic acid using the same procedure for the preparation **2**. Compound **4** was prepared by the catalytic hydrogenation of **2j** (Scheme 2).

3. Pharmacology

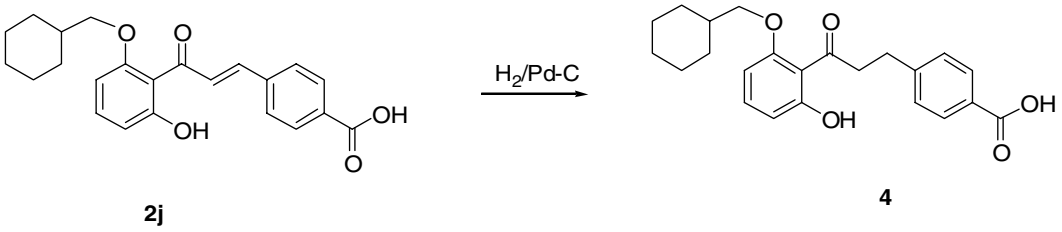
The measurement of inhibitory activity of IL-5 of these analogs was performed with the comparison of cell proliferation of Y16 cell with/without the samples.[18] An Y16 cell line originated from murine early B cell is proliferated in the presence of IL-5, which was used as the parameter of IL-5 bioactivity. Non-radioactive procedures that measure cell metabolism as an index of proliferation have now become popular. 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium sodium salt (WST-1) was used to form soluble formazan product on exposure to the dehydrogenase activity in metabolizing cells. Proliferation of Y16 cells in the presence of IL-5 was specifically blocked by treatment of polyclonal antibody against IL-5 or monoclonal antibody against IL-5Rα. Sample and IL-5 were simultaneously added to the Y16 cells, and the bioassay system can identify the IL-5 antagonist and its signal inhibitor. The results of biological screening of chalcone analogs against IL-5 are listed in Table 1 as % inhibition at 50 μM and IC_{50} values.

4. Results and discussion

Considering the activity of budesonide (70.3% inhibition at 50 μM, IC_{50} = 26.8 μM) being used for the treatment of chronic asthma, the activities of **2b** (78.8% inhibition at 50 μM, IC_{50} = 25.3 μM) and **2d** (77.6% inhibition at 50 μM, IC_{50} = 28.8 μM) appear to be very potent. To explore structure–activity relationship of **2**, benzyloxy group was initially varied. Omission of benzyloxy group as shown in **2a** and **2o** reduces the activity. Cyclohexylmethoxy analog **2e** (99.0% inhibition at 50 μM, IC_{50} = 12.6 μM) shows two times more potency than 2-benzyloxy analog **2b**. The same trend appears in



Scheme 1. Preparation of chalcone analogs.



Scheme 2. Reduction of **2j**.

comparison of 4-cyclohexylmethoxy analog **2g** and 4-benzyoxy analog **2d**. Regardless of their positions on A ring, the hydrophobic groups on A ring obviously produce favorable effect for the enhancement of activity of these series. Compounds **2b** and **2e** containing 2-alkoxy group have slightly better activity than **2d** and **2e** possessing 4-alkoxy group. Thus 2-alkoxy-6-hydroxy analogs **2** were mostly prepared and tested.

Substituents at 4-position of B ring greatly influence the change of activity of **2**. Comparison of the activities of **2j** (4-carboxylic acid analog), **2m** (3-carboxylic acid analog), and **2n** (2-carboxylic acid analog) indicates that carboxylic acid at 4-position of B ring most enhances the activity. Therefore, 4-substituted derivatives of **2** were mainly investigated. Replacement of hydroxyl group of **2b** or **2e** with electron withdrawing group capable to be hydrogen bonding acceptor such as carboxylic acid (**2h** and **2j**), carboxylic ester (**2k**), aldehyde (**2v**), chloro (**2q**), and sulfonamide (**2w**, **2x**) increases the activity. The activities of compounds **2e**, **2j**, **2k**, **2q**, **2s**, **2u**, **2v**, and **2w** are well correlated with Hammet parameter (σ_p)[29] as shown in Eq. 1. In case of **2j**, σ_p value (0) for carboxylate anion was used for finding equation 1 as a real form of carboxylic acid under the bioassay condition.

$$\log 1/IC_{50} = 0.763\sigma_p - 0.86(n = 8, R^2 = 0.86) \quad (1)$$

Despite σ_p values (0) of H in **2o** and ethyl in **2t** being the same as those for COO^- in **2h** and $NHCOCH_3$ in **2s**, the activities of **2o** (99.6% inhibition at 50 μM, IC_{50} = 14.3 μM) and **2t** (82.3% inhibition at 50 μM, IC_{50} = 27.9 μM) are much weaker than those of **2j** and **2s**. These differences imply that substituents at 4-position of B ring of **2** should have strong hydrogen acceptor property in hydrogen bonding in addition to large electron withdrawing property for the potent inhibitory activity against IL-5 induced proliferation of eosinophil. The activity of **2k** with methyl ester was much more enhanced compared to that of the corresponding carboxylic acid analog **2j**. Methyl ester has larger σ_p compared to caboxylate anion and hydrogen acceptor property without ability of hydrogen donor. Thus this also supports that these electron withdrawing and hydrogen acceptor properties of substituents are important for the activity of **2**.

The size of substituents at 4-position of B ring should be important factor for the activity of **2**. Comparison of the activities of hydroxyl analogs **2b** and **2e** and their methoxmethoxy analogs **2c** (47.8% inhibition at 50 μM, IC_{50} > 50 μM) and **2f** (13.0% inhibition at 50 μM, IC_{50} > 50 μM) reveals that methoxymethoxy group dramatically reduces the activity. Weaker activities of **2c** and **2f** than those of **2b** and **2e** might indicate that methoxymethoxy group may be too large to fit with putative binding pocket regardless of its hydrogen acceptor property. In spite of its incapability in hydrogen bonding, ethyl analog **2t** (82.3% inhibition at 50 μM, IC_{50} = 27.9 μM) has fairly strong activity. Hydroxymethyl analog **2u** (99.3% inhibition at 50 μM, IC_{50} = 6.4 μM) with hydrogen bonding capability possesses the more potent activity. Therefore the substituents at 4-position of B ring should be as small as three atom size.

To identify the role of hydroxyl group on A ring of **2**, compound **3** without hydroxyl group on A ring was prepared. This compound shows very weak activity (15.6% inhibition at 50 μM, IC_{50} > 50 μM). Therefore hydroxyl function on A ring of **2** is essential for the activity.

To determine the role of propenone system for the activity of **2**, compound **2j** was converted to its propanone analog **4** by the catalytic hydrogenation. Inhibitory activity of **4** (15.0% inhibition at 50 μM, IC_{50} > 50 μM) against IL-5 was abolished. Thus the α,β-unsaturated ketone of **2** is critical for the activity.

5. Conclusion

Novel chalcones were prepared and identified as potent inhibitors of IL-5 bioactivity. Among them, compound **2w** (4-[(*E*)-3-(2-(cyclohexylmethoxy)-6-hydroxyphenyl)-3-oxoprop-1-enyl]benzenesulfonamide) is the most active derivative (99.5% inhibition at 50 μM, IC_{50} = 1.8 μM). The structural requirement of chalcone analogs possessing the potent inhibitory activity against IL-5 could be summarized as the following. (1) The hydrophobic group such as benzyloxy or cyclohexylmethoxy at 2-position of A ring is necessary. (2) The existence of phenolic hydroxyl at 6-position of A ring is critical. (3) Propenone motif as α,β-unsaturated ketone is essential. (4) Electron withdrawing groups with hydrogen acceptor property at 4-position of B ring as small as three-atom size enhance the activity. The quantitative structure–activity relationship of **2** regarding these substituents was well determined.

6. Experimental

6.1. Chemistry

Melting points (mp) were determined on Electrothermal 1A 9100 MK2 apparatus and are uncorrected. All commercial chemicals were used as obtained and all solvents were purified by the standard procedures prior to use.[30] Thin-layer chromatography was performed on E Merck silica gel GF-254 precoated plates and the identification was done with UV light and colorization with spray 10% phosphomolybdic acid followed by heating. Flash column chromatography was performed with E. Merck silica gel (230-400mesh). IR spectra were recorded with Jasco IR-Report-100 IR spectrometer in cm^{-1} and corrected against peak at 1601 cm^{-1} of polystyrene. ^1H NMR spectra were measured against the peak of tetramethylsilane by Varian Unity Inova 400 NMR (400 MHz) spectrometers. Elemental analysis was performed with EA1110 elemental analyzer (CE Instrument).

6.2. General procedure[20] for the preparation of compounds (5)

2,6-Dihydroxyacetophenone or 2,4-dihydroxyacetophenone (40 mmol) was dissolved in dry acetonitrile (80 mL) and potassium carbonate (6.624 g, 48 mmol)

was added. The resulting mixture was refluxed for 1 h and then cyclohexylmethyl methanesulfonate (9.216 g, 48 mmol) in acetonitrile (10 mL) was dropped for fifteen min. The reaction mixture was refluxed for 1.5 h. After cooling to room temperature, dichloromethane (320 mL) was added and washed with 5% aqueous sodium hydroxide and water three times. The organic layer was dried with anhydrous sodium sulfate and concentrated under vacuum. The crude product was purified by flash column chromatography.

6.2.1. 2-Cyclohexylmethoxy-6-hydroxyacetophenone (5a). Yield 71.0%; yellow solid; mp 71.3–72.4 °C; R_f 0.30 (hexanes/ethyl acetate = 20:1). IR (KBr) 3420, 3050, 2900, 1620 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.07–1.33 (m, 5H), 1.68–2.02 (m, 6H), 2.71 (s, 3H), 3.83 (d, J = 5.6, 2H), 6.36 (d, J = 8.4 Hz, 1 H), 6.54 (d, J = 8.4 Hz, 1H), 7.31 (t, J = 8.4 Hz, 1H), 7.68 (s, 1H); Anal. Calcd for $C_{15}H_{20}O_3$: C, 72.55; H, 8.12. Found: C, 72.36; H, 8.09.

6.2.2. 2-Cyclohexylmethoxy-6-hydroxyacetophenone (5b). Yield 67.9%; yellow solid; mp 73.0–74.2 °C; R_f 0.32 (hexanes/ethyl acetate = 20:1). IR (KBr) 3400, 3030, 2850, 1620 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.19–1.29 (m, 5H), 1.69–1.87 (m, 6H), 2.72 (s, 3H), 3.81 (d, J = 6.0, 2H), 6.40 (d, J = 8.4 Hz, 1H), 6.53 (d, J = 8.4 Hz, 1H), 7.29 (t, J = 8.4 Hz, 1H), 7.57 (s, 1H); Anal. Calcd for $C_{15}H_{20}O_3$: C, 72.55; H, 8.12. Found: C, 72.40; H, 8.07.

6.2.3. 2-Benzyloxyacetophenone (5c). Using the method for the preparation of **3a**, **3c** was prepared from 2-hydroxybenzaldehyde and benzyl bromide.

Yield 63.2%; yellow solid; mp 92.2–93.4 °C; R_f 0.32 (hexanes/ethyl acetate = 10:1). IR (KBr) 3030, 2930, 1620 cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.62 (s, 3H), 5.13 (s, 2H), 6.47 (d, J = 8.4 Hz, 1H), 6.59(d, J = 8.4 Hz, 1H), 7.33(t, J = 8.4 Hz, 1H), 7.41 (m, 5H); Anal. Calcd for $C_{15}H_{14}O_2$: C, 79.62; H, 6.24. Found: C, 79.42; H, 6.17.

6.3. General procedure for the preparation of compounds 2

The corresponding 2-hydroxyacetophenone **5** (1 equiv) was added to 90% aqueous ethanol solution of two equivalents of sodium hydroxide or potassium hydroxide (8.7% w/w). The substituted benzaldehyde **6** (1.02 equiv) was then added and the resulting solution was stirred for 2 h at 50 °C. After removal of ethanol under vacuum, the crude mixture was dissolved in water and neutralized with hydrochloric acid. The resulting mixture was extracted with dichloromethane three times. The combined organic layers were dried with anhydrous sodium sulfate and evaporated under vacuum. The crude product was purified by flash column chromatography to give **2**. In case of preparation of **2a**, **2b**, **2d**, **2e**, and **2g**, the corresponding intermediates 1-(2-hydroxyphenyl)-3-[4-(methoxymethoxy)phenyl]propenones such as **2c** or **2f** were hydrolyzed in hydrochloric acid in aqueous methanol (90%) by reflux for 2 h to convert 1-(2-hydroxyphenyl)-3-(4-hydroxyphenyl)propenones **2**.

6.3.1. (*E*)-3-(4-Hydroxyphenyl)-1-(2-hydroxyphenyl)-2-propen-1-one (2a). Yield 93.8%, orange solid; mp 133.5–134.7 °C; R_f 0.20 (hexanes/ethyl acetate = 3:1). IR (KBr) 3300, 3100, 2950, 1640 cm^{-1}. ^1H NMR ($CDCl_3$) δ 6.90 (d, J = 8.4 Hz, 1H), 6.98 (m, 2H), 7.50 (t, J = 8.4 Hz, 1H), 7.54 (d, J = 15.6 Hz, 1H), 7.60 (d, J = 8.4 Hz, 2H), 7.89 (d, J = 15.6 Hz, 1H), 7.92 (d, J = 6.4 Hz, 2 H), 12.92 (s, 1H); Anal. Calcd for $C_{15}H_{12}O_3$: C, 74.99; H, 5.03. Found: C, 74.73; H, 4.97.

6.3.2. (*E*)-1-(2-Benzyloxy-6-hydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one (2b). Yield 86.4%, orange solid; mp 143.2–144.3 °C; R_f 0.26 (hexanes/ethyl acetate = 3:1). IR (KBr) 3400, 3100, 2950, 1640 cm^{-1}. ^1H NMR ($CDCl_3$) δ 5.19 (s, 2H), 6.69–6.89 (m, 3H), 7.25 (d, J = 8.6 Hz, 1H), 7.38 (d, J = 8.8 Hz, 2H), 7.50 (m, 6H), 7.76 (d, J = 15.6 Hz, 2H), 13.64 (s, 1H); Anal. Calcd for $C_{22}H_{18}O_4$: C, 76.29; H, 5.24. Found: C, 76.18; H, 5.15.

6.3.3. (*E*)-1-(4-Benzyloxy-2-hydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one (2d). Yield 81.2%; brown solid; mp 164.1–165.0 °C; R_f 0.14 (hexanes/ethyl acetate = 5:1). IR (KBr) 3400, 3080, 2850, 1630 cm^{-1}. ^1H NMR ($CDCl_3$) δ 5.11 (s, 2H), 6.55 (d, J = 8.4 Hz, 1H), 6.59 (s, 1H), 6.93 (m, 3H), 7.35 (d, J = 8.8 Hz, 2H), 7.40 (m, 5H), 7.43 (d, J = 15.2 Hz, 1H), 7.86 (d, J = 15.2 Hz, 1H), 13.54 (s, 1H); Anal. Calcd for $C_{22}H_{18}O_4$: C, 76.29; H, 5.24. Found: C, 75.99; H, 5.14.

6.3.4. (*E*)-1-(2-Cyclohexylmethoxy-6-hydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one (2e). Yield 52.9%; yellow solid; mp 173.7–174.4 °C; R_f 0.31 (hexanes/ethyl acetate = 3:1). IR (KBr) 3400, 3100, 2850, 1620 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.07–1.26 (m, 6H), 1.64–1.72 (m, 5H), 3.86 (d, J = 6.4, 2H), 6.40 (d, J = 8.4 Hz, 1H), 6.87 (d, J = 8.4 Hz, 2H), 7.32 (t, J = 8.4 Hz,1H), 7.52 (d, J = 8.4 Hz, 2H), 7.78 (d, J = 15.6 Hz, 1H), 7.84 (d, J = 15.6 Hz, 1H), 13.27(s, 1H); Anal. Calcd for $C_{22}H_{24}O_4$: C, 74.98; H, 6.86. Found: C, 74.79; H, 6.77.

6.3.5. (E)-1-(2-Cyclohexylmethoxy-6-hydroxyphenyl)-3-(4-methoxymethoxyphenyl)-2-propen-1-one (2f). Yield 71.1%, yellow solid; mp 90.4–91.7 °C; R_f 0.36 (hexanes/ethyl acetate = 7:1). IR (KBr) 3350, 3100, 2950, 1630 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.06–1.25 (m, 5H), 1.63–1.71 (m, 3H), 1.87–1.90 (m, 3H), 3.49 (s, 3H), 3.86 (d, J = 5.6 Hz, 2H,), 5.22 (s, 2H), 6.40 (d, J = 7.6 Hz, 1H), 6.59 (d, J = 7.6 Hz, 1H), 7.06 (d, J = 8.8 Hz, 2H), 7.32 (t, J = 8.4 Hz, 1H), 7.56 (d, J = 8.8 Hz, 2H), 7.79 (d, J = 15.6 Hz, 1H), 7.85 (d, J = 15.6 Hz, 1H), 12.97 (s, 1H); Anal. Calcd for $C_{24}H_{28}O_5$: C, 72.70; H, 7.12. Found: C, 72.56; H, 6.98.

6.3.6. (*E*)-1-(4-Cyclohexylmethoxy-2-hydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one (2g). Yield 50.9%, yellow solid; mp 171.8–172.4 °C; R_f 0.26 (hexanes/ethyl acetate = 3:1). IR (KBr) 3400, 3100, 2900, 1640 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.19–1.29 (m, 5H), 1.69–1.87 (m, 6H), 3.81 (d, J = 6.0 Hz, 2H), 6.46 (m, 2H), 6.88 (d, J = 8.8 Hz, 2 H), 7.45 (d, J = 15.2 Hz, 1H), 7.57 (d, J = 8.8 Hz, 2H), 7.79 (s 1H), 7.84 (d, J = 15.2 Hz, 1H),

13.07(s, 1H); Anal. Calcd for $C_{22}H_{24}O_4$: C, 74.98; H, 6.86. Found: C, 74.83; H, 6.75.

6.3.7. 4-[(*E*)-3-(2-Benzyloxy-6-hydroxyphenyl)-3-oxopropen-1-yl]benzoic acid (2h). Yield 68.7%, yellow solid; mp 187.5–188.1 °C; R_f 0.33 (hexanes/ethyl acetate = 1:2). IR (KBr) 3400, 3100, 2950, 1690, 1630 cm^{-1}. ^1H NMR ($CDCl_3$) δ 5.12 (s, 2H), 6.51 (d, J = 8.4 Hz, 1H), 6.69 (d, J = 8.4 Hz, 1H), 7.07 (t, J = 8.4 Hz, 1H), 7.16 (d, J = 8.8 Hz, 1H), 7.32 (d, J = 8.8 Hz, 2H), 7.44 (m, 6H), 7.75 (d, J = 15.2 Hz, 1H), 7.85 (d, J = 15.2 Hz, 1H), 10.02 (s, 1H), 13.03(s, 1H); Anal. Calcd for $C_{23}H_{18}O_5$: C, 73.79; H, 4.85. Found: C, 73.65; H, 4.73.

6.3.8. 4-[(*E*)-3-(4-Benzyloxy-2-hydroxyphenyl)-3-oxopropen-1-yl]benzoic acid (2i). Yield 37.9%; yellow solid; mp 236.1–237.2 °C; R_f 0.46 (hexanes/ethyl acetate = 1:2). IR (KBr) 3400, 3100, 2930, 1700, 1650 cm^{-1}. ^1H NMR (DMSO-d_6) δ 5.23 (s, 2H), 6.62 (s, 1H), 6.66 (d, J = 7.1, 1H), 7.42 (d, J =8.8 Hz, 2H), 7.69 (d, J = 15.2 Hz, 1H), 7.92 (d, J = 7.1 Hz, 1H), 7.99 (d, J = 15.2 Hz, 1H), 8.05 (d, J = 8.8 Hz, 2H), 10.60 (s, 1H), 13.17(s, 1H); Anal. Calcd for $C_{23}H_{18}O_5$: C, 73.79; H, 4.85. Found: C, 73.64; H, 4.79.

6.3.9. 4-[(*E*)-3-(2-Cyclohexylmethoxy-6-hydroxyphenyl)-3-oxopropen-1-yl]benzoic acid (2j). Yield 49.2%; yellow solid; mp 213.8–214.9 °C; R_f 0.35 (hexanes/ethyl acetate = 1:1). IR (KBr) 3400, 3100, 2950, 1680, 1630 cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.01 (m, 5H), 1.54 (m, 5H), 2.51 (m, 1H), 3.80 (d, J = 5.6 Hz, 1H), 6.54 (d, J = 8.2 Hz, 2H), 7.24 (d, J = 8.2 Hz, 1H), 7.38 (d, J = 8.2 Hz, 2H), 7.75 (d, J = 15.2 Hz, 1H), 7.84 (d, J = 8.2 Hz, 2H), 8.05 (d, J = 15.2 Hz, 1H), 10.80 (s, 1H), 13.07 (s, 1H); Anal. Calcd for $C_{23}H_{24}O_5$: C, 72.61; H, 6.36. Found: C, 72.49; H, 6.24.

6.3.10. 4-[(*E*)-3-(4-Cyclohexylmethoxy-2-hydroxyphenyl)-3-oxopropen-1-yl]benzoic acid (2l). Yield 55.9%; yellow solid; mp 249.4–250.2 °C; R_f 0.46 (hexanes/ethyl acetate = 1:1). IR (KBr) 3400, 3100, 2950, 2850, 1680, 1640 cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.15 (m, 5H), 1.71 (m, 6H), 3.87 (d, J = 5.7 Hz, 2H), 6.51 (d, J = 8.2 Hz, 2H), 7.61 (d, J = 8.3 Hz, 1H), 7.83 (d, J = 8.2 Hz, 2H), 7.91 (d, J = 15.2 Hz, 1H), 8.01 (d, J = 8.2 Hz, 2H), 8.23 (d, J = 15.2 Hz, 1H), 10.84 (s, 1H), 13.31 (s, 1H); Anal. Calcd for $C_{23}H_{24}O_5$: C, 72.61; H, 6.36. Found: C, 72.47; H, 6.25.

6.3.11. 3-[(*E*)-3-(2-(Cyclohexylmethoxy)-6-hydroxyphenyl)-3-oxopropen-1-yl]benzoic acid (2m). Yield 60.0%; yellow solid; mp 188.2–189.1 °C; R_f 0.46 (hexanes/ethyl acetate = 2:1). IR (KBr) 3370, 3100, 2900, 2850, 1690, 1640 cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.01–1.08 (m, 5H), 1.48–1.68 (m, 6H), 3.80 (d, J = 6.0 Hz, 2H), 6.52 (d, J = 8.8 Hz, 1H), 6.55 (d, J = 8.8, 1H), 7.28 (t, J = 8.8 Hz, 1H), 7.41 (d, J = 16.0 Hz, 1H), 7.51 (d, J =8.2 Hz, 2H), 7.55 (d, J = 7.6 Hz, 1H), 7.95 (m, 2H), 8.18 (s, 1H), 13.15 (s, 1H); Anal. Calcd for $C_{23}H_{24}O_5$: C, 72.61; H, 6.36. Found: C, 72.51; H, 6.31.

6.3.12. 2-((*E*)-3-(2-(Cyclohexylmethoxy)-6-hydroxyphenyl)-3-oxopropen-1-yl)benzoic acid (2n). Yield 67.7%; yellow solid; mp 176.3–177.2 °C; R_f 0.37 (hexanes/ethyl acetate = 1:1). IR (KBr) 3410, 3100, 2930, 2850, 1700, 1640 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.00–1.14 (m, 6H), 1.63–1.71 (m, 5H), 3.80 (s, 2H), 6.09 (t, J = 8.0 Hz, 1H), 6.51 (d, J = 8.4 Hz, 2H), 7.29 (t, J = 8.4 Hz, 1H), 7.60 (t, J = 8.4 Hz, 1H), 7.69 (d, J = 15.6 Hz, 1H), 7.77 (m, 2H), 7.84 (d, J = 15.6 Hz, 1H), 13.02 (s, 1H); Anal. Calcd for $C_{23}H_{24}O_5$: C, 72.61; H, 6.36. Found: C, 72.58; H, 6.27.

6.3.13. (*E*)-4-[3-(2-Hydroxyphenyl)-3-oxopropen-1-yl]benzoic acid (2o). Yield 90.0%; yellow solid; mp 248.5–252.0 °C; R_f 0.21 (hexanes/ethyl acetate = 1:2). IR (KBr) 3400, 3100, 2950, 1690, 1630 cm^{-1}. ^1H NMR (DMSO-d_6) δ 7.01 (m, 2H), 7.58 (t, J = 7.6 Hz, 1H), 7.85 (d, J = 15.6 Hz, 1H), 8.01 (m, 4H), 8.12 (d, J = 15.6, Hz, 1H), 8.23 (d, J = 15.6 Hz, 1H), 8.23 (d, J =8.0 Hz, 1H), 12.30 (s, 1H), 13.04 (s, 1H); Anal. Calcd for $C_{16}H_{12}O_4$: C ,71.64; H, 4.51. Found: C, 71.45; H, 4.46.

6.3.14. (*E*)-1-[2-(Cyclohexylmethoxy)-6-hydroxyphenyl]-3-phenylprop-2-en-1-one (2p). Yield 58.3%; orange solid; mp 94.2–96.4 °C; R_f 0.21 (hexanes/ethyl acetate = 10:1). IR (KBr) 3400, 3100, 2950, 1640 cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.01–1.23 (m, 5H), 1.59–1.89 (m, 6H), 3.84 (d, J = 6.0 Hz, 2H), 6.39 (d, J = 8.0 Hz, 1H), 6.59 (d, J = 8.4 Hz, 1H), 7.32 (t, J = 8.4 Hz, 1H), 7.40 (m, 4H), 7.60 (m, 2H), 7.79 (d, J = 15.6 Hz, 1H), 7.94 (d, J = 15.6 Hz, 1H), 12.84 (s, 1H); Anal. Calcd for $C_{22}H_{24}O_3$: C, 78.54; H, 7.19. Found: C, 78.45; H, 7.07.

6.3.15. (*E*)-3-(4-Chlorophenyl)-1-[2-(cyclohexylmethoxy)-6-hydroxyphenyl]-2-propen-1-one (2q). Yield 53%, orange solid; mp 120.7–122.6 °C; R_f 0.29 (hexanes/ethyl acetate = 10:1). IR (KBr) 3400, 3100, 2950, 1640 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.01–1.21 (m, 5H), 1.59–1.88 (m, 6H), 3.85 (d, J = 6.0 Hz, 2H), 6.39 (d, J = 8.4 Hz, 1H), 6.59 (d, J = 8.4 Hz, 1H), 7.33 (t, J = 8.4 Hz, 1H), 7.37 (d, J = 8.4 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H), 7.72 (d, J = 15.6 Hz, 1H), 7.90 (d, J = 15.6 Hz, 1H), 13.04 (s, 1H); Anal. Calcd for $C_{22}H_{23}ClO_3$: C, 71.25; H, 6.25. Found: C, 71.12; H, 6.17.

6.3.16. (*E*)-*N*-4-{3-[2-(Cyclohexylmethoxy)-6-hydroxyphenyl]-3-oxoprop-1-enyl}phenyl acetamide (2s). Yield 64.2%; yellow solid; mp 203.0–204.0 °C; R_f 0.21 (hexanes/ethyl acetate = 6:1). IR (KBr) 3300, 3100, 2950, 1680, 1640 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.05–1.25 (m, 5H), 1.63–1.70 (m, 3H), 1.87–1.89 (m, 3H), 2.21 (s, 3H), 3.85 (d, J = 6.0 Hz, 2H), 6.39 (d, J = 8.4 Hz, 1H), 6.58 (d, J = 8.4 Hz, 1H), 7.32 (t, J = 8.4 Hz, 1H), 7.44 (s, 1H), 7.56 (s, 4H), 7.75 (d, J = 15.6 Hz, 1H), 7.88 (d, J = 15.6 Hz, 1H), 13.02 (s, 1H); Anal. Calcd for $C_{24}H_{27}O_4$: C, 73.26; H, 6.92. Found: C, 73.14; H, 6.84.

6.3.17. (*E*)-1-(2-(Cyclohexylmethoxy)-6-hydroxyphenyl)-3-(4-ethylphenyl)-2-propen-1-one (2t). Yield 69.3%; orange solid; mp 81.0–82.1 °C; R_f 0.29 (hexanes/ethyl acetate = 10:1). IR (KBr) 3400, 3100, 2950, 1640 cm^{-1}. NMR ($CDCl_3$) δ 1.03–1.27 (m, 8H), 1.66 (t, J = 14.4 Hz, 3H), 1.88 (m, 3H), 2.68 (q, J = 7.6 Hz, 2H), 3.85 (d, J = 5.6 Hz, 2H), 6.39 (d, J = 8.4 Hz, 1H),

6.58 (d, J = 8.4 Hz, 1H), 7.22 (d, J =8.0 Hz, 2H), 7.31 (t, J = 8.4 Hz, 1H), 7.52 (d, J = 8.0 Hz, 2H), 7.79 (d, J = 15.6 Hz, 1H), 7.91 (d, J = 15.6 Hz, 1H), 12.95 (s, 1H); Anal. Calcd for $C_{24}H_{28}O_3$: C, 79.09; H, 7.74. Found: C, 78.86; H, 7.65.

6.3.18. (*E*)-1-(2-(Cyclohexylmethoxy)-6-hydroxyphenyl)-3-(4-(hydroxymethyl)phenyl)-2-propen-1-one (2u). Yield 66.2%; orange solid; mp 93.3–94.5 °C; R_f 0.45 (hexanes/ethyl acetate = 2:1). IR (KBr) 3300, 2930, 2850, 1640 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.03–1.25 (m, 6H), 1.67–1.90 (m, 5H), 3.87 (s, 3H), 4.75 (s, 2H), 6.39 (d, J = 8.4 Hz, 1H), 6.59 (d, J = 8.4 Hz, 1H), 7.33 (t, J = 8.4 Hz, 1H), 7.40 (d, J = 8.0 Hz, 2H), 7.59 (d, J = 8.0 Hz, 2H), 7.79 (d, J = 15.6 Hz, 1H), 7.95 (d, J = 15.6 Hz, 1H), 13.20 (s, 1H); Anal. Calcd for $C_{23}H_{26}O_4$: C, 75.38; H, 7.15. Found: C, 75.25; H, 7.07.

6.3.19. (*E*)-4-{3-[2-(Cyclohexylmethoxy)-6-hydroxyphenyl]-3-oxoprop-1-enyl}benzalde hyde (2v). Yield 60.5%; orange solid; mp 116.0–117.2 °C; R_f 0.19 (hexanes/ethyl acetate = 8:1). IR (KBr) 3420, 3100, 2950, 1700, 1640 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.00–1.18 (m, 5H), 1.59–1.87 (m, 6H), 3.86 (d, J = 6.0 Hz, 2H), 6.40 (d, J = 8.4 Hz, 1H), 6.60 (d, J = 8.4 Hz, 1H), 7.36 (t, J = 8.4 Hz, 1H), 7.74 (d, J = 8.4 Hz, 2 H), 7.77 (d, J = 15.6 Hz, 1H), 7.92 (d, J = 8.4 Hz, 2H), 8.01 (d, J = 15.6 Hz, 1H), 10.10 (s, 1H), 13.02 (s, 1H); Anal. Calcd for $C_{23}H_{24}O_4$: C, 75.80; H, 6.64. Found: C, 75.67; H, 6.48.

6.3.20. 4-((*E*)-3-(2-(Cyclohexylmethoxy)-6-hydroxyphenyl)-3-oxoprop-1-enyl)benznesulfonamide (2w). Yield 56.2%; yellow solid; mp 130.1–131.3 °C; R_f 0.33 (hexanes/ethyl acetate = 2:1). IR (KBr) 3420, 3400, 3100, 1640 cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.01–1.10 (m, 5H), 1.52–1.99 (m, 6H), 3.79 (d, J = 5.6 Hz, 2H), 6.53 (d, J = 80 Hz, 1H), 6.56 (d, J = 8.4 Hz, 1H), 7.26 (t, J = 8.4 Hz, 1H), 7.39 (d, J = 15.6 Hz, 2H), 7.45 (s, 2H), 7.82 (d, J = 8.4 Hz, 1H), 7.88 (d, J = 8.4 Hz, 1H), 10.63 (s, 1H); Anal. Calcd for $C_{22}H_{25}NO_5S$: C, 63.59; H, 6.06. Found: C, 63.42; H, 5.97.

6.3.21. 4-((*E*)-3-(2-(Benzyloxy)-6-hydroxyphenyl)-3-oxoprop-1-enyl)benzenesulfonamide (2x). Yield 52.4%; yellow solid; mp 182.0–183.2 °C; R_f 0.33 (hexanes/ethyl acetate = 2:1). IR (KBr) 3420, 3400, 3100, 1640 cm^{-1}. ^1H NMR (DMSO-d_6) δ 5.13 (s, 2H), 6.57 (d, J = 8.4 Hz, 1H), 6.68 (d, J = 8.4 Hz, 1H), 7.26 (t, J = 8.4 Hz, 1H), 7.36 (d, J = 8.4 Hz, 2H), 7.44–7.72 (m, 7H), 7.78 (d, J = 15.6 Hz, 1H), 7.82 (d, J = 15.6 Hz, 1H), 10.65 (s, 1H); Anal. Calcd for $C_{22}H_{19}NO_5S$: C, 64.53; H, 4.68. Found: C, 64.39; H, 4.57.

6.4. Preparation of (*E*)-methyl 4-{3-[2-(cyclohexylmethoxy)-6-hydroxyphenyl]-3-oxoprop-1-enyl}benzoate (2k)

Compound **2j** (0.5 g, 1.3 mmol) and sulfuric acid 1 mL were dissolved in methanol (50 mL) and then the reaction mixture was refluxed for 2 h. After cooling, the mixture was concentrated under vacuum. The residue was dispersed in dichloromethane and washed with cold water three times and 10% aqueous sodium bicarbonate. The organic layer was dehydrated with anhydrous sodium sulfate and concentrated under vacuum. The crude product was purified by flash column chromatography to give **2k**.

Yield 40%, yellow solid; mp 118.7–119.5 °C; R_f 0.22 (hexanes/ethyl acetate = 8:1). IR (KBr) 3450, 3100 2950, 1720, 1630 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.04–1.19 (m, 5H), 1.65–1.69 (m, 3H), 1.85–1.88 (m, 3H), 3.86 (d, J = 5.6 Hz, 2H) 3.95 (s, 3H), 6.40 (d, J = 8.4 Hz, 1H), 6.60 (d, J = 6.4 Hz, 1H), 7.34 (t, J = 8.4 Hz, 1H), 7.65 (d, J = 8.4 Hz, 2H), 7.77 (d, J = 8.4 Hz, 1H), 7.37 (d, J = 15.6 Hz, 1H), 7.98 (d, J = 15.6 Hz, 1H), 8.07 (d, J = 8.4 Hz, 2H), 13.07 (s, 1H); Anal. Calcd for $C_{24}H_{26}O_5$: C, 73.08; H, 6.64. Found: C, 72.88; H, 6.53.

6.5. Preparation of (*E*)-3-(4-aminophenyl)-1-[2-(cyclohexylmethoxy)-6-hydroxyphenyl]-2-propen-1-one (2r)

Hydrochloric acid (2 N, 10 mL) was added to the solution of **2s** (0.27 g, 0.69 mmol) in ethanol (50 mL). The resulting mixture was refluxed for 3 h and concentrated under vacuum. The crude mixture was dispersed in dichloromethane (50 mL) and basified with saturated aqueous sodium bicarbonate solution. The organic layer was separated, dehydrated with anhydrous sodium sulfate, and concentrated under vacuum. The crude product was purified by flash column chromatography.

Yield 75.2%; brown solid; mp 122.7–128.5 °C; R_f 0.20 (hexanes/ethyl acetate = 3:1). IR (KBr) 3450, 3200, 2900, 1620 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.00–1.19 (m, 5H), 1.58–1.67 (m, 3H), 1.83–1.97 (m, 3H), 3.77 (d, J = 6.0 Hz, 2H), 3.94 (s, 1H), 6.31 (d, J = 8.4 Hz, 1H), 6.50 (d, J = 8.4 Hz, 1H), 6.59 (d, J = 8.4 Hz, 2H), 7.22 (t, J = 8.4 Hz, 1H), 7.37 (d, J = 8.4 Hz, 2H), 7.73 (s, 2H), 12.94 (s, 1H); Anal. Calcd for $C_{22}H_{25}O_3$: C, 75.19; H, 7.17. Found: C, 75.03; H, 7.02.

6.6. Preparation of 4-[(*E*)-3-(2-benzyloxyphenyl)-3-oxopropen-1-yl]benzoic acid (3)

Using the method for the preparation of **2a**, compound **3** was synthesized from 1-(2-(benzyloxy))acetophenone.

Yield 57.3%; yellow solid; mp 140.1–141.5 °C; R_f 0.48 (hexanes/ethyl acetate = 2:1). IR (KBr) 2930, 2850, 1640 cm^{-1} . ^1H NMR ($CDCl_3$) δ 5.16 (s, 2H), 7.06–7.11 (m, 2H), 7.31–7.44 (m, 8H), 7.34 (d, J = 15.6 Hz, 1H), 7.40 (d, J = 15.6 Hz, 1H), 7.51 (t, J = 8.4 Hz, 1H), 8.01 (d, J = 8.4 Hz, 2H), 12.10 (s, 1H); Anal. Calcd for $C_{23}H_{24}O_4$: C, 75.80; H, 6.64. Found: C, 75.62; H, 6.48.

6.7. Preparation of 4-{3-[2-(cyclohexylmethoxy)-6-hydroxyphenyl]-3-oxopropyl} benzoic acid (4)

Compound **2j** (200 mg, mmol) was dissolved in methanol (50 mL) and 10% Pd/C (20 mg) was added. The resulting mixture was stirred under hydrogen (20 psi) for two hours at room temperature. After removal of catalyst by filtration with aid of Celite pad, the solvent

was removed under vacuum. The crude product was purified by flash column chromatography.

Yield 77%, white solid; mp 198.2–199.4 °C; R_f 0.45 (hexanes/ethyl acetate = 4:1). IR (KBr) 3300, 2930, 2850, 1720, 1690 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.96–1.12 (m, 5H), 1.64–1.77 (m, 6H), 3.11 (t, J = 8.0 Hz, 2H), 3.51 (t, J = 8.0 Hz, 2H), 3.81 (d, J = 5.6 Hz, 2H), 6.36 (d, J = 8.4 Hz, 1H), 6.56 (d, J = 8.4 Hz, 1H), 7.31 (t, J = 8.4 Hz, 1H), 7.34 (d, J = 8.4 Hz, 2H), 8.04 (d, J = 8.4 Hz, 2H), 9.88 (s, 1H), 12.01 (s, 1H); Anal. Calcd for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85. Found: C, 72.18; H, 6.80.

6.8. Inhibitory activity against interleukin-5

The Y16 cell line was grown in RPMI (10.4 mg/mL RPMI-1640, 24 mM $NaHCO_3$, 100 U/mL benzylpenicillin potassium, and 100 μg/mL streptomycin sulfate, pH 7.1) containing 8% fetal bovine serum (FBS) and 5 U/mL IL-5 at 37 °C with 5% CO_2. The grown Y16 cells were harvested by centrifugation at 250*g* for 10 min at 4 °C, washed twice with Hanks' solution (9.8 mg/mL Hanks' balanced salts, 24 mM $NaHCO_3$, pH 7.1), and resuspended in a small volume of RPMI containing 8% FBS. Number of the cells were counted after trypan blue exclusion and then diluted to 1×10^5 cells/mL with RPMI containing 8% FBS. Viability of the cells was more than 95% in all preparations. One hundred microliters of 1×10^4 cells was dispensed to each well of a 96-well microplate (Nunc, Denmark), and 50 μL of 4 U/mL IL-5 and 50 μL of the sample were added. Control group was treated with RPMI containing 8% FBS instead of sample, and blank group with RPMI containing 8% FBS instead of IL-5. After incubation at 37 °C with 5 CO_2 for 48 h, Y16 cells in each well were treated with 20 μL WST-1 solution (3.3 mg WST-1 and 0.7 mg 1-methoxy-5-methyl-phenazinium methylsulfate per mL of phosphate-buffered saline) and incubated 37 °C with 5% CO_2 for 4 h. Absorbance at wavelength 450 nm (A_{450}) was measured by using a microplate reader (Molecular Device, USA). Inhibitory effect on the IL-5 bioassay by sample at 50 μM concentration was expressed as % inhibition [1-(sample A_{450} − blank A_{450})/(control A_{450} − blank A_{450})] × 100. Data were collected as means ± SEM of three independent tests. Samples showing more than 50% inhibition at 50 μM concentration were serially diluted and then subjected to the IL-5 bioassay to determine IC_{50} values.

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