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Short communication

Synthesis, biological evaluation of chrysin derivatives as potential immunosuppressive agents

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ABSTRACT

A series of novel chrysin derivatives was firstly synthesized and evaluated on their immunosuppressive activity in the search for potential immunosuppressive agents. Among them, compounds **5c** displayed the most potent immunosuppressive inhibitory activity with IC_{50} of 0.78 μM, which was comparable to that of cyclosporin A (IC_{50} = 0.06 μM). The preliminary mechanism of compound **5c** inhibition effects was also detected by flow cytometry (FCM), and the compound exerted immunosuppressive activity via inducing the apoptosis of activated lymph node cells in a dose dependent manner. Furthermore, the estimated LD_{50} (in mg/kg) in vivo of compound **5c** is 738.2, which indicated that compound **5c** was low toxic.

1. Introduction

Immunosuppressant is an important class of clinical drugs for an array of medical processes, including transplant rejection and the treatment of autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and psoriasis [1–3]. Although immunosuppressive drugs have been used for organ transplantation and treatment of autoimmune diseases in clinic, their side effects including liver toxicity, nephrotoxicity, infection, cardiovascular toxicity and others can not be neglected [4–9]. Therefore, there is a clinical need for new therapeutic agents with anti-inflammatory activity. A program to produce synthetic compounds is ongoing in our laboratory to identify new immunosuppressants [10].

Flavonoids are natural polyphenolic phytochemicals that are ubiquitous in plants and present in the average human diet. Chrysin (5,7-dihydroxyflavone, **1**) is a natural flavonoid found in many plant extracts, honey, and propolis, which has been reported to have many different biological activities such as anti-viral [11], anti-cancer [12], anti-bactericidal [13], anti-inflammatory [14], anti-allergic [15], DNA cleavage [16], vasodilator [17], anti-mutagenic [18], anti-anxiolytic [19] and anti-oxidant [20] effects. Recently, Eun Kyung Shin et al., reported that chrysin could alleviate the symptoms of dextran sodium sulfate (DSS)-induced colitis in mice by reducing the production of inflammatory mediators, which suggesting that chrysin exerts potentially clinically useful anti-inflammatory effects [21].

As we know, salicylic acid was identified in willow bark extracts as an active anti-inflammatory compound over a century ago. It is reported that some salicylic acid derivatives showed potent anti-inflammatory activity [22–24]. For example, 5-aminosalicylic acid (5-ASA) was an active ingredient of agents used for the long term maintenance therapy to prevent relapses of inflammatory bowel diseases [25]. Besides, the methyl and some other esters of salicylic acids and their derivatives were found to have much lower gastric ulcerogenic activity compared with their corresponding acids [26–28]. In view of the facts mentioned above, and in continuation to extend our research on new potential immunosuppressive agents with high efficacy and low toxicity [10], we turned our attention to natural product chrysin. Therefore, a series of novel substituted salicylic acid and chrysin derivatives were firstly synthesized. Compound **5c** displayed potent immunosuppressive activity by inhibiting CD3/CD28 co-stimulated T cell proliferation, suggesting that it may have a characteristic to inhibit the T-cell mediated immune response. Subsequently, the function of compound **5c** on the cell apoptosis in activated mouse T cells was studied.

2. Results and discussion

2.1. Chemistry

In this study, twenty novel substituted salicylic acid and chrysin derivatives were synthesized. The synthetic route of them is shown in Scheme 1. To a stirring solution of chrysin (CR, compound **1**) in

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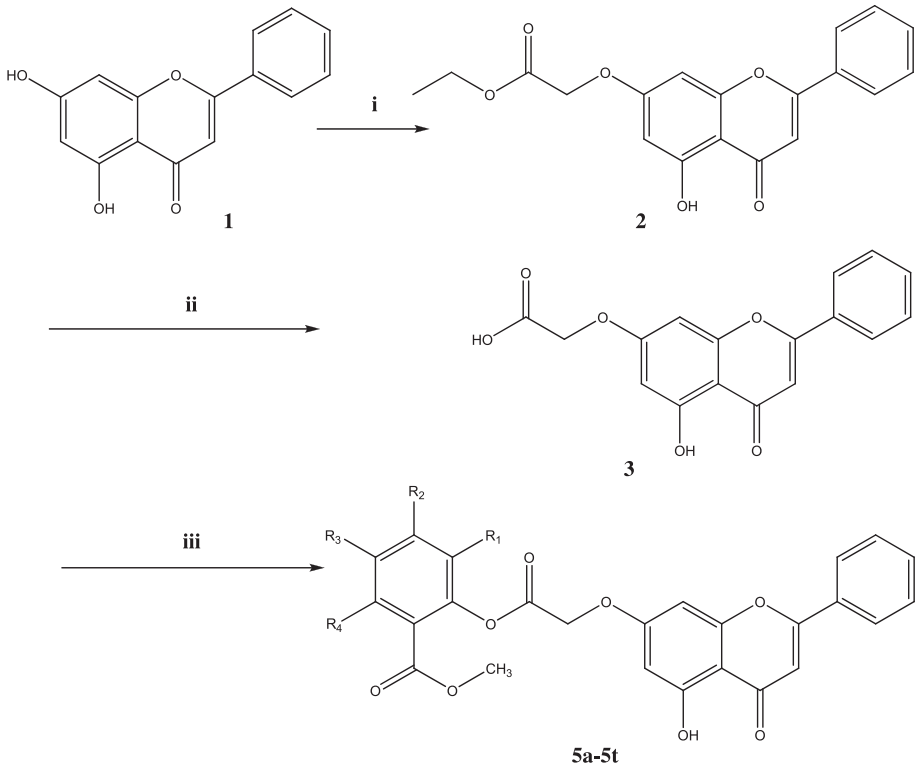

5a R_1= H, R_2= H, R_3= OH, R_4= H
5b R_1= H, R_2= H, R_3= CH_3, R_4= H
5c R_1= H, R_2= H, R_3= Br, R_4= H
5d R_1= H, R_2= H, R_3= Cl, R_4= H
5e R_1= H, R_2= H, R_3= F, R_4= H
5f R_1= H, R_2= CH_3, R_3= H, R_4= H
5g R_1= H, R_2= Cl, R_3= H, R_4= H
5h R_1= H, R_2= Br, R_3= H, R_4= H
5i R_1= OCH_3, R_2= H, R_3= H, R_4= H
5j R_1= H, R_2= H, R_3= H, R_4= H
5k R_1= Br, R_2= H, R_3= H, R_4= H
5l R_1= Cl, R_2= H, R_3= H, R_4= H
5m R_1= CH_3, R_2= H, R_3= H, R_4= H
5n R_1= H, R_2= Cl, R_3= H, R_4= Cl
5o R_1= H, R_2= Br, R_3= H, R_4= Br
5p R_1= H, R_2= H, R_3= H, R_4= F
5q R_1= H, R_2= H, R_3= H, R_4= Cl
5r R_1= H, R_2= H, R_3= H, R_4= Br
5s R_1= H, R_2= H, R_3= H, R_4= CH_3
5t R_1= H, R_2= H, R_3= H, R_4= OH

Scheme 1. General synthesis of compounds **5a**–**5t**. Reagents and conditions: i) $BrCH_2COOEt$, K_2CO_3, acetone; ii) Na_2CO_3, 5% aq, DMSO; iii) substituted methyl salicylate (**4a**–**4t**), EDC, HOBt, DMAP, DMF, 10–12 h.

acetone, K_2CO_3 was added and then a mixture of ethyl bromoacetate and acetone was added dropwise to give compound **2**. Compound **2** was treated in Na_2CO_3 solution (DMSO) and after that most of the volatiles were evaporated. The residue was dissolved in water and the solution was adjusted to pH 2 by using HCl solution to give compound **3**. Compound **3** was suspended in DMF with stirring at room temperature, then substituted methyl salicylate (**4a**–**4t**), 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC.HCl), *N*-Hydroxybenzotriazole (HOBt) and 4-dimethylaminopyridine (DMAP) were added. The reaction solution was stirred for 12 h at room temperature. Then, compounds **5a**–**5t** were obtained by subsequent purification with flash chromatography. All of the synthetic compounds gave satisfactory analytical and spectroscopic data, which were in full accordance with their depicted structures.

2.2. Immunosuppressive activity

T cells play a pivotal role in immune response. Excessive T-cell proliferation and activation has been implicated in the pathogenesis of a variety of inflammatory diseases.

The new synthetic chrysin derivatives were tested in vitro for their cytotoxicity on lymph node cells and inhibition activity on CD3/CD28 co-stimulated lymph node cells with cyclosporin A (CsA) as the control. The pharmacological results of these compounds were summarized in Table 1. The cytotoxicity of each compound was expressed as the concentration of compound that reduced cell viability to 50% (CC_{50}). The immunosuppressive activity of each compound was expressed as the concentration of the compound that inhibited CD3/CD28 co-stimulated T cell proliferation to 50% (IC_{50}) of the control value. The selective index (SI) value was used to evaluate the bioactivity of the compounds.

Studies were performed by modification of the phenyl ring of the salicylic acid derivatives to determine how the substituents of the subunits affected the immunosuppressive activity. As shown in Table 1, in general, most of the synthesized novel chrysin derivatives exhibited higher immunosuppressive activity than the parent compound chrysin (IC_{50}: 44.16 μM), with IC_{50} ranging from 0.78 μM to 45.72 μM. Among them, compounds with substitutions on the R_1- and R_4- position of phenyl ring of the salicylic acid derivatives (IC_{50}: from 36.78 μM to 45.72 μM) showed less potent immunosuppressive activity than the R_2- and R_3- position ones (IC_{50}: from 0.78 μM to 31.07 μM). For the R_2- position substituted ones, replacement of H atom at R2-position by one halogen atom (**5g** IC_{50}: 12.36 μM and **5h** IC_{50}: 17.59 μM) resulted in the improving of their immunosuppressive activity compared to **5f** (IC_{50}: 31.07 μM).

Table 1
In vitro cytotoxicity on lymph node cells and inhibitory effects of the synthetic compounds **5a**–**5t** on lymph node cells co-stimulated by CD3/CD28.

Compd [a]	CC_{50} (μM)	IC_{50} (μM)	SI [b]
CR	428.27	44.16	9.70
5a	263.42	29.47	8.94
5b	306.48	25.68	11.93
5c	>450	0.78	>576.92
5d	>436	3.74	>116.57
5e	389.26	6.82	50.08
5f	249.81	31.07	8.04
5g	264.83	12.36	21.43
5h	326.18	17.59	18.54
5i	196.34	36.78	5.34
5j	264.92	28.54	9.28
5k	378.63	40.65	9.31
5l	366.24	38.74	9.45
5m	298.86	39.08	7.65
5n	345.26	43.57	7.92
5o	397.64	45.72	8.69
5p	318.72	40.27	7.91
5q	365.84	38.94	9.39
5r	387.91	41.72	9.28
5s	297.85	37.16	8.02
5t	337.69	38.25	8.83
CsA	15.82	0.06	263.67

[a] The compounds tested for immunosuppressive activity were consistent with the description in the Experimental Section.
[b] Selectivity index [SI] is determined as the ratio of the concentration of the compound that reduced cell viability to 50% (CC_{50}) to the concentration of the compound needed to inhibit the proliferation to 50% (IC_{50}) of the control value.

For compounds **5n** and **5o**, introduced two halogen on the R2-position and R4- position, the immunosuppressive activity was remarkably decreasing (**5n** IC_{50}: 43.57 μM and **5o** IC_{50}: 45.72 μM) compared to compounds **5g** and **5h**. Besides, the SI values of the compounds differed greatly, ranging from 5.34 to more than 576.92. Some of the synthesized compounds (**5g** and **5h**), especially **5g** (SI = 21.43) showed good immunosuppressive activity but much less potent than CsA (SI = 263.67).

It is clear that the synthesized compounds with halogen substitutions in R_3- position on the phenyl ring of the salicylic acid derivatives (compounds **5c**, **5d**, and **5e**) showed higher activity than others, with IC_{50} ranging from 0.78 μM to 6.82 μM. Among them, compounds **5c** displayed the most potent inhibitory activity with IC_{50} of 0.78 μM, which was comparable to that of cyclosporin A (IC_{50} = 0.06 μM). More significantly, compounds **5c** exhibited reduced cytotoxicity by over 100-fold compared with cyclosporin A (CsA) and more potent inhibition activity (SI > 576.92) than CsA (SI = 263.67). Therefore, the IC_{50} and SI value suggest that compounds **5c** may be a promising lead for the further development of novel therapeutic agents.

Apoptosis is an essential mechanism used to eliminate activated T cells during the shutdown process of excess immune responses and maintain proper immune homeostasis, while deficient apoptosis of activated T cells is associated with a wide variety of immune disorders. As a representative of these chrysin derivatives, compound **5c** has been under investigations in vitro experiment. We detected the mechanism of compound **5c** inhibition effects by flow cytometry (FCM) (Fig. 1), and found that the compound could induce the apoptosis of activated lymph node cells in a dose dependent manner. As shown in Fig. 1, lymph node cells stimulated with anti-CD3/anti-CD28 were treated with 1, 2, 4 and 8 μM of compound **5c** for 48 h. The compound increased the percentage of apoptosis by Annexin V-FITC/PI staining in a dose-dependent manner. The result indicated that compound **5c** induced apoptosis of anti-CD3/anti-CD28 stimulated lymph node cells.

Our attention in this area is to search for new potential immunosuppressive agents with high efficacy and low toxicity, so the acute oral toxicity test of the most potent compound **5c** was examined. It can be seen from Table 2 that the estimated LD_{50} (in mg/kg) in vivo of compound **5c** is 738.2, which indicated that compound **5c** was low toxic. Furthermore, we also evaluate the effect of chrysin derivative **5c** on three human tumor cell lines: human hepatocellular liver carcinoma (Hep-G2), human erthro-myeloblastoid leukemia (K562) and human oral epidermoid carcinoma (KB), and the normal human liver cell line (L02), and the in vitro cytotoxicity results were showed in Table 3. Compound **5c** exhibited significant inhibitory activity against K562, KB and Hep-G2 with IC_{50} of 0.47, 0.79 and 0.61 μM, respectively. Besides, Compound **5c** also displayed little toxicity against the normal human liver cell line (L02) with IC_{50} of 274 μM.

3. Conclusions

In summary, a series of novel chrysin derivatives were firstly synthesized and evaluated for their immunosuppressive activity on CD3/CD28 co-stimulated T cell proliferation. It is worth noting that compound **5c** displayed the most outstanding immunosuppressive effects *in vitro* with IC_{50} of 0.78 μM, which was comparable to that of cyclosporin A (IC_{50} = 0.06 μM). Moreover, the preliminary mechanism of compound **5c** inhibition effects was also detected by flow cytometry (FCM), and the compound exerted immunosuppressive activity via inducing the apoptosis of activated lymph node cells in a dose dependent manner. In addition, the acute oral toxicity test demonstrated that compound **5c** was low toxic. All the results outlined the great potential of compound **5c** for further exploitation as immunosuppressant.

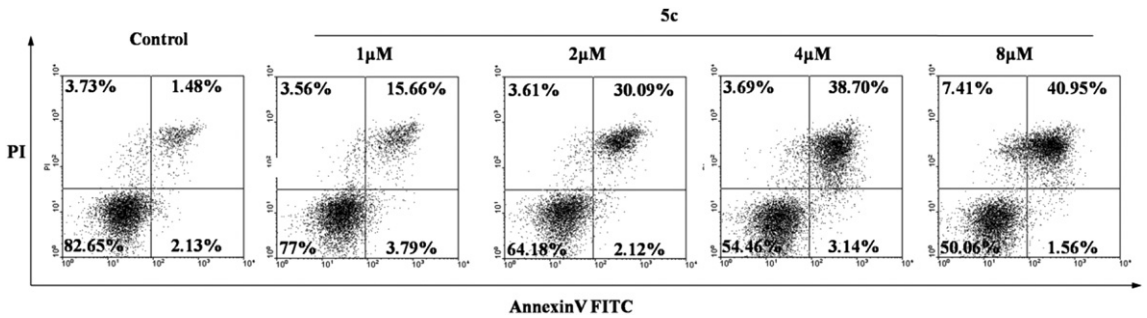

Fig. 1. Lymph node cells isolated from naive mice were cultured with anti-CD3/anti-CD28 and various concentrations of **5c** for 48 h. Cells were stained by Annexin V–FITC/PI and apoptosis was analyzed by flow cytometry.

Table 2
Estimated LD_{50} (in mg/kg) in vivo of compound **5c**.

Compound	Estimated LD_{50} [a] (mg/kg)
5c	738.2

[a] The LD_{50} is calculated with AOT425StatPgm based on maximum likelihood for long term results at 95% confidence interval.

Table 3
In vitro cytotoxicity (IC_{50} value, μM) on three human tumor cell lines human hepatocellular liver carcinoma (Hep-G2), human erthromyeloblastoid leukemia (K562) and human oral epidermoid carcinoma (KB), and the normal human liver cell line (L02) of compound **5c**.

Compound	K562	KB	Hep-G2	L02
5c	0.47 ± 0.06	0.79 ± 0.18	0.61 ± 0.13	274 ± 26
5-fluorouracil	38 ± 7	2.16 ± 0.22	2.78 ± 0.31	56 ± 9

4. Experimental Section

4.1. General

Separation of the compounds by column chromatography was carried out with silica gel 60 (200–300 mesh ASTM, E. Merck). The quantity of silica gel used was 50–100 times the weight charged on the column. Then, the eluates were monitored using TLC. Melting points (uncorrected) were determined on an XT4 MP apparatus (Taike Corp., Beijing, China). ESI mass spectra were obtained on a Mariner System 5304 mass spectrometer, and ^{1}H NMR spectra were recorded on a Bruker PX500 or DPX300 spectrometer at 25 °C with TMS and solvent signals allotted as internal standards. Chemical shifts were reported in ppm (δ). Elemental analyses were performed on a CHN-O-Rapid instrument and were within ±0.4% of the theoretical values.

4.2. Biological assay

4.2.1. Animals

Specific-pathogen-free female BALB/c mice, 6–8 week-old, were obtained from Experimental Animal Center of Yangzhou University (Yangzhou, China). They were maintained in plastic cages at 22 ± 2 °C and kept on a 12 h light–dark cycle with free access to pellet food and water. Animal welfare and experimental procedures were carried out strictly in accordance with the guide for the care and use of laboratory animals (National Research Council of USA, 1996). All efforts were made to minimize animals' suffering and to reduce the number of animals used.

4.2.2. Reagents and materials

T cells were incubated in RPMI 1640 medium supplemented with 100 U mL^{-1} of penicillin, 100 μgm L^{-1} of streptomycin and 10% fetal calf serumunder a humidified 5% (v/v) CO_2 atmosphere at 37 °C. Chrysin (purity>98%, obtained from the Shanxi Huike co., Shanxi, China) is dissolved at a concentration of 0.05 mol/L in 100% DMSO as a stock solution, stored at −20 °C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study (all the control groups are composed of 0.1% DMSO). Other drugs and reagents used in this study are as follows: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO); Cyclosporin A (CsA, Sigma Chemical Co., St. Louis, MO); Concanavalin A (Con A, Sigma Chemical Co., St. Louis, MO); Annexin V-FITC/PI Kit (Jingmei Biotech, Nanjing, China).

4.2.3. Cell proliferation assay

Cells were incubated in 96-well plate at a density of 5×10^5 cells per well and stimulated with anti-CD3/anti-CD28 in the presence of various concentrations of compounds for 72 h. For proliferation assay, 20 μL MTT (Sigma, MO; 4 mg/mL in PBS) was added per well 4 h before the end of the incubation. After removing the supernatant, 200 μL DMSO was added to dissolve the formazan crystals. The absorbance at 540 nm (OD540) was read on an ELISA reader (Tecan, Austria).

4.2.4. Cytotoxicity test

Cells were incubated in 96-well plate at a density of 5×10^5 cells per well with various concentrations of compounds for 48 h. For cytotoxicity assay, 20 μL of MTT (Sigma, MO; 4 mg/mL in PBS) was added per well 4 h before the end of the incubation. MTT formazan production was dissolved by 200 μL DMSO replacing the medium. The absorbance was read on an ELISA reader (Tecan, Austria) at 540 nm (OD540).

4.2.5. Apoptosis assay

Lymph node cells were stimulated with anti-CD3/anti-CD28 in the presence of various concentrations of compounds for 48 h and then stained with both Annexin V–FITC (fluorescein isothiocyanate) and propidium iodide (PI). Then samples were analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

4.2.6. Acute oral Toxicity of compound ***5c***

Healthy nulliparous and non-pregnant BALB/C female mice, 12 weeks of age at the initiation of the experiment were used. All animals were fastening prior to dosing by withholding food (not water) for 3–4 h and 1–2 h after dosing. After that period access to food and water was ad libitum. Animals were kept in conventional circumstances: light/dark rhythms 12/12 h, temperature 22 °C, and humidity 55%. All experiments were performed according to the OECD 425 Guideline for the testing of chemicals—Acute oral Toxicity: Up-and-down procedure [29]. Altogether 35 animals were used in experiments to determine LD_{50} for compound **5c**. Animals are dosed, one at a time, at 24 h intervals. The first animal receives a dose at the level of the best estimate of the LD_{50}. Depending on the outcome for the previous animal, the dose for the next animal is adjusted up or down. If an animal survives, the dose for the next animal is increased; if it dies, the dose for the next animal is decreased. After reaching the reversal of the initial outcome, that is, the point where an increasing (or decreasing) dose pattern is reversed by giving a smaller (or a higher) dose, four additional animals are dosed following the same UDP.

4.2.7. Statistical analysis

Statistical analyses were conducted by the 'Acute Oral Toxicity (Guideline 425) Statistical Program' (AOT425StatPgm) which is developed by Westat for the US EPA and designed to be used with the acute oral toxicity testing procedure presented in OECD Guideline for the testing of chemicals, Section 4: Health Effects Test No. 425, Acute Oral Toxicity: Up-and-Down Procedure [29].

4.3. Chemistry

4.3.1. General procedure for the preparation of compound ***2***

A solution of compound **1** (2.84 g, 11.2 mmol), ethyl bromoacetate (3.8 g, 22.7 mmol), and powdered potassium carbonate (3 g, 22 mmol) in acetone (50 mL) was refluxed for 48 h (the reaction was monitored by TLC). After cooling, removal of the solvent under reduced pressure gave a crude product, which was filtered off and purified by washing with water (50 mL), NaOH (1 M, 30 mL), and

water again (30 mL) to give the product **2** as a yellow powder. Yield: 86%. M.p.: 277–279 °C ^{1}H NMR (300 MHz, d_6-DMSO): 1.25 (t, J = 6.9 Hz, 3H); 4.19 (m, 2H); 4.96 (s, 2H); 6.43 (m, 1H); 6.85 (m, 1H), 7.01 (s, 1H), 7.60 (m, 3H), 8.10 (m, 2H), 12.81 (s, 1H). MS (ESI): 340.1 ($C_{19}H_{17}O_6$, $[M + H]^+$). Anal. Calcd for $C_{19}H_{16}O_6$: C, 67.05; H, 4.74%; Found: C, 67.18; H, 4.91%.

*4.3.2. General procedure for the preparation of compound **3***

A solution of sodium carbonate (5% aqueous, 8 mL, excess) was added to a solution of compound **2** (0.98 g, 2.82 mmol) in DMSO (50 mL). The mixture was refluxed for 48 h (the reaction was monitored by TLC). After cooling, removal of the solvent under reduced pressure, and acidification with HCl (1 M) gave a crude product, which was filtered off and purified by washing with water (50 mL) to give the product **3** as a yellow powder. Yield: 80%. M.p.: 296–298 °C; ^{1}H NMR (500 MHz, d_6-DMSO): 2.51 (s, 3H); 4.85 (s, 2H); 6.41 (m, 1H), 6.82 (m, 1H); 7.04 (s, 1H); 7.61 (m, 3H), 8.11 (m, 2H), 12.80 (s, 1H), 13.20 (s, 1H). MS (ESI): 312.1 ($C_{17}H_{13}O_6$, $[M + H]^+$). Anal. Calcd for $C_{17}H_{12}O_6$: C, 65.39; H, 3.87%; Found: C, 65.17; H, 3.73%.

*4.3.3. General procedure for the preparation of compounds **5a**–**5t***

A solution of compound **3**, substituted methyl salicylate (**4a**–**4t**), 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC.HCl), *N*-Hydroxybenzotriazole (HOBt) and 4-dimethylaminopyridine (DMAP) in DMF (50 mL) was stirred for 12 h at room temperature. Then, compounds **5a**–**5t** were obtained by subsequent purification with flash chromatography (acetate: petroleum ether = 1:5–1:2).

*4.3.3.1. Methyl 5-hydroxy-2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoate (**5a**).* White powder. Yield: 74%. M.p.: 187–188 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.86 (s, 3H); 5.31 (s, 2H); 6.53–6.56 (d, J = 2.17 Hz, 1H); 6.94–6.97 (d, J = 2.43 Hz, 1H); 7.08 (s, 1H); 7.41–7.43 (d, J = 5.67 Hz, 1H); 7.57–7.62 (m, 3H); 7.91–7.94 (dd, J_1 = 2.47, J_2 = 8.56 Hz, 1H); 7.94–7.96 (d, J = 2.61 Hz, 1H); 8.11–8.13 (m, 2H); 12.82 (s, 1H). MS (ESI): 463.1 ($C_{25}H_{19}O_9$, $[M + H]^+$). Anal. Calcd for $C_{25}H_{18}O_9$: C, 64.94; H, 3.92%; Found: C, 64.76; H, 3.74%.

*4.3.3.2. Methyl 2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)-5-methylbenzoate (**5b**).* White powder. Yield: 71%. M.p.: 196–197 °C ^{1}H NMR (300 MHz, d_6-DMSO): 2.38 (s, 3H); 3.85 (s, 3H); 5.31 (s, 2H); 6.53–6.55 (d, J = 2.06 Hz, 1H); 6.96–6.97 (d, J = 2.43 Hz, 1H); 7.07 (s, 1H); 7.41–7.43 (d, J = 5.61 Hz, 1H); 7.57–7.62 (m, 3H); 7.93–7.96 (dd, J_1 = 2.41, J_2 = 8.61 Hz, 1H); 7.95–7.96 (d, J = 2.73 Hz, 1H); 8.09–8.11 (m, 2H); 12.81 (s, 1H). MS (ESI): 461.1 ($C_{26}H_{21}O_8$, $[M + H]^+$). Anal. Calcd for $C_{26}H_{20}O_8$: C, 67.84; H, 4.38%; Found: C, 67.62; H, 4.59%.

*4.3.3.3. Methyl 5-bromo-2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoate (**5c**).* White powder. Yield: 76%. M.p.: 202–203 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.86 (s, 3H); 5.28 (s, 2H); 6.54–6.55 (d, J = 2.09 Hz, 1H); 6.96–6.97 (d, J = 2.43 Hz, 1H); 7.06 (s, 1H); 7.34–7.36 (d, J = 5.13 Hz, 1H); 7.57–7.63 (m, 3H); 7.92–7.95 (dd, J_1 = 2.55, J_2 = 8.61 Hz, 1H); 8.07–8.08 (d, J = 2.71 Hz, 1H); 8.09–8.11 (m, 2H); 12.80 (s, 1H). ^{13}C NMR (d_6-DMSO): 54.6, 65.2, 93.5, 98.7, 104.6, 106.2, 114.8, 117.4, 122.6, 126.1, 127.7, 127.9, 128.8, 129.1, 129.8, 139.2, 148.5, 156.7, 160.6, 162.3, 164.7, 171.4, 184.9, 191.4. MS (ESI): 525.0 ($C_{25}H_{18}BrO_8$, $[M + H]^+$). Anal. Calcd for $C_{25}H_{17}BrO_8$: C, 57.16; H, 3.26%; Found: C, 57.41; H, 3.38%.

*4.3.3.4. Methyl 5-chloro-2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoate (**5d**).* White powder. Yield: 73%. M.p.: 204–205 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.87 (s, 3H); 5.30 (s, 2H); 6.55–6.56 (d, J = 2.19 Hz, 1H); 6.98–6.99 (d, J = 2.40 Hz, 1H); 7.08 (s, 1H); 7.42–7.44 (d, J = 5.58 Hz, 1H); 7.59–7.64 (m, 3H); 7.92–7.95 (dd, J_1 = 2.57, J_2 = 8.64 Hz, 1H); 7.96–7.97 (d, J = 2.73 Hz, 1H); 8.10–8.12 (m, 2H); 12.83 (s, 1H). MS (ESI): 481.0 ($C_{25}H_{18}ClO_8$, $[M + H]^+$). Anal. Calcd for $C_{25}H_{17}ClO_8$: C, 62.45; H, 3.56%; Found: C, 62.73; H, 3.35%.

*4.3.3.5. Methyl 5-fluoro-2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoate (**5e**).* White powder. Yield: 75%. M.p.: 207–209 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.84 (s, 3H); 5.27 (s, 2H); 6.54–6.56 (d, J = 2.17 Hz, 1H); 6.94–6.96 (d, J = 2.41 Hz, 1H); 7.07 (s, 1H); 7.36–7.38 (d, J = 5.17 Hz, 1H); 7.59–7.64 (m, 3H); 7.91–7.94 (dd, J_1 = 2.64, J_2 = 8.73 Hz, 1H); 8.08–8.09 (d, J = 2.73 Hz, 1H); 8.08–8.10 (m, 2H); 12.81 (s, 1H). MS (ESI): 465.0 ($C_{25}H_{18}FO_8$, $[M + H]^+$). Anal. Calcd for $C_{25}H_{17}FO_8$: C, 64.66; H, 3.69%; Found: C, 64.37; H, 3.46%.

*4.3.3.6. Methyl 2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy) acetoxy)-4-methylbenzoate (**5f**).* White powder. Yield: 70%. M.p.: 183–184 °C ^{1}H NMR (300 MHz, d_6-DMSO): 2.39 (s, 3H); 3.83 (s, 3H); 5.27 (s, 2H); 6.54–6.55 (d, J = 1.97 Hz, 1H); 6.98–6.99 (d, J = 2.43 Hz, 1H); 7.06 (s, 1H); 7.17 (s, 1H); 7.25–7.27 (d, J = 7.74 Hz, 1H); 7.59–7.62 (m, 3H); 7.87–7.89 (d, J = 4.74 Hz, 1H); 8.09–8.11 (m, 2H); 12.82 (s, 1H). MS (ESI): 461.1 ($C_{26}H_{21}O_8$, $[M + H]^+$). Anal. Calcd for $C_{26}H_{20}O_8$: C, 67.82; H, 4.38%; Found: C, 67.63; H, 4.52%.

*4.3.3.7. Methyl 4-chloro-2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoate (**5g**).* White powder. Yield: 71%. M.p.: 171–174 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.82 (s, 3H); 5.26 (s, 2H); 6.53–6.54 (d, J = 2.14 Hz, 1H); 6.97–6.98 (d, J = 2.41 Hz, 1H); 7.06 (s, 1H); 7.18 (s, 1H); 7.26–7.28 (d, J = 7.81 Hz, 1H); 7.58–7.63 (m, 3H); 7.88–7.89 (d, J = 4.71 Hz, 1H); 8.09–8.13 (m, 2H); 12.81 (s, 1H). MS (ESI): 481.0 ($C_{25}H_{18}ClO_8$, $[M + H]^+$). Anal. Calcd for $C_{25}H_{17}ClO_8$: C, 62.45; H, 3.56%; Found: C, 62.73; H, 3.71%.

*4.3.3.8. Methyl 4-bromo-2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoate (**5h**).* White powder. Yield: 74%. M.p.: 176–178 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.84 (s, 3H); 5.27 (s, 2H); 6.52–6.54 (d, J = 2.19 Hz, 1H); 6.96–6.97 (d, J = 2.43 Hz, 1H); 7.06 (s, 1H); 7.16 (s, 1H); 7.27–7.29 (d, J = 7.83 Hz, 1H); 7.56–7.61 (m, 3H); 7.86–7.88 (d, J = 4.74 Hz, 1H); 8.08–8.12 (m, 2H); 12.83 (s, 1H). MS (ESI): 525.0 ($C_{25}H_{18}BrO_8$, $[M + H]^+$). Anal. Calcd for $C_{25}H_{17}BrO_8$: C, 57.16; H, 3.26%; Found: C, 57.43; H, 3.47%.

*4.3.3.9. Methyl 2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)-3-methoxybenzoate (**5i**).* White powder. Yield: 73%. M.p.: 180–182 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.87 (s, 3H); 3.89 (s, 3H); 5.30 (s, 2H); 6.92–6.93 (d, J = 2.40 Hz, 1H); 7.07 (s, 1H); 7.37–7.51 (m, 3H); 7.58–7.64 (m, 3H); 8.09–8.12 (m, 2H); 12.84 (s, 1H). MS (ESI): 477.1 ($C_{26}H_{21}O_9$, $[M + H]^+$). Anal. Calcd for $C_{26}H_{20}O_9$: C, 65.55; H, 4.23%; Found: C, 65.72; H, 4.43%.

*4.3.3.10. Methyl 2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoate (**5j**).* White powder. Yield: 76%. M.p.: 174–175 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.89 (s, 3H); 5.30 (s, 2H); 6.56–6.57 (d, J = 2.07 Hz, 1H); 6.99–7.00 (d, J = 2.41 Hz, 1H); 7.07 (s, 1H); 7.35–7.37 (d, J = 7.95 Hz, 1H); 7.45–7.48 (m, 1H); 7.58–7.63 (m, 3H); 7.72–7.75 (m, 1H); 7.98–8.01 (dd, J_1 = 2.43, J_2 = 8.67 Hz, 1H); 8.10–8.12 (m, 2H); 12.83 (s, 1H). MS (ESI): 447.1 ($C_{25}H_{20}O_8$, $[M + H]^+$). Anal. Calcd for $C_{25}H_{18}O_8$: C, 67.26; H, 4.06%; Found: C, 67.47; H, 4.31%.

*4.3.3.11. 3-Bromo-2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoic acid (**5k**).* White powder. Yield: 70%. M.p.: 163–165 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.88 (s, 3H); 5.31 (s, 2H);

6.91–6.93 (d, J = 2.42 Hz, 1H); 7.09 (s, 1H); 7.45–7.53 (m, 3H); 7.59–7.66 (m, 3H); 8.02–8.11 (m, 2H); 12.83 (s, 1H). MS (ESI): 511.1 ($C_{24}H_{16}BrO_8$, $[M + H]^+$). Anal. Calcd for $C_{24}H_{15}BrO_8$: C, 56.38; H, 2.96%; Found: C, 56.41; H, 2.89%.

*4.3.3.12. 3-Chloro-2-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoic acid (**5l**).* White powder. Yield: 73%. M.p.: 168–169 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.87 (s, 3H); 5.30 (s, 2H); 6.94–6.99 (d, J = 2.47 Hz, 1H); 7.07 (s, 1H); 7.51–7.58 (m, 3H); 7.63–7.69 (m, 3H); 8.06–8.17 (m, 2H); 12.86 (s, 1H). MS (ESI): 467.1 ($C_{24}H_{16}ClO_8$, $[M + H]^+$). Anal. Calcd for $C_{24}H_{15}ClO_8$: C, 61.75; H, 3.24%; Found: C, 61.69; H, 3.29%.

*4.3.3.13. 2-(2-(5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy) acetoxy)-3-methylbenzoic acid (**5m**).* White powder. Yield: 71%. M.p.: 171–173 °C ^{1}H NMR (300 MHz, d_6-DMSO): 2.36 (s, 3H); 3.84 (s, 3H); 5.32 (s, 2H); 6.87–6.95 (d, J = 2.41 Hz, 1H); 7.13 (s, 1H); 7.39–7.46 (m, 3H); 7.51–7.62 (m, 3H); 8.14–8.21 (m, 2H); 12.81 (s, 1H). MS (ESI): 447.1 ($C_{25}H_{19}O_8$, $[M + H]^+$). Anal. Calcd for $C_{25}H_{18}O_8$: C, 67.26; H, 4.06%; Found: C, 67.19; H, 4.12%.

*4.3.3.14. 2,4-Dichloro-6-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoic acid (**5n**).* White powder. Yield: 68%. M.p.: 192–193 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.81 (s, 3H); 5.28 (s, 2H); 6.56–6.64 (d, J = 2.34 Hz, 1H); 6.84–6.89 (d, J = 2.47 Hz, 1H); 7.08 (s, 1H); 7.13 (s, 1H); 7.27–7.34 (d, J = 7.69 Hz, 1H); 7.54–7.61 (m, 2H); 7.86–7.92 (d, J = 4.76 Hz, 1H); 8.09–8.14 (m, 2H); 12.84 (s, 1H). MS (ESI): 501.1 ($C_{24}H_{15}Cl_2O_8$, $[M + H]^+$). Anal. Calcd for $C_{24}H_{14}Cl_2O_8$: C, 57.51; H, 2.82%; Found: C, 57.63; H, 2.89%.

*4.3.3.15. 2,4-Dibromo-6-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoic acid (**5o**).* White powder. Yield: 73%. M.p.: 207–209 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.83 (s, 3H); 5.26 (s, 2H); 6.52–6.60 (d, J = 2.31 Hz, 1H); 6.78–6.84 (d, J = 2.56 Hz, 1H); 7.11 (s, 1H); 7.19 (s, 1H); 7.31–7.37 (d, J = 7.56 Hz, 1H); 7.51–7.58 (m, 2H); 7.76–7.83 (d, J = 4.71 Hz, 1H); 8.06–8.12 (m, 2H); 12.83 (s, 1H).MS (ESI): 588.1 ($C_{24}H_{15}Br_2O_8$, $[M + H]^+$). Anal. Calcd for $C_{24}H_{14}Br_2O_8$: C, 48.84; H, 2.39%; Found: C, 48.75; H, 2.45%.

*4.3.3.16. 2-Fluoro-6-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoic acid (**5p**).* White powder. Yield: 70%. M.p.: 185–187 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.86 (s, 3H); 5.33 (s, 2H); 6.86–6.91 (d, J = 2.47 Hz, 1H); 7.11 (s, 1H); 7.47–7.56 (m, 3H); 7.65–7.73 (m, 2H); 8.09–8.17 (m, 2H); 12.81 (s, 1H). MS (ESI): 451.1 ($C_{24}H_{16}FO_8$, $[M + H]^+$). Anal. Calcd for $C_{24}H_{15}FO_8$: C, 64.00; H, 3.36%; Found: C, 64.08; H, 3.41%.

*4.3.3.17. 2-Chloro-6-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoic acid (**5q**).* White powder. Yield: 72%. M.p.: 190–192 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.87 (s, 3H); 5.30 (s, 2H); 6.74–6.87 (d, J = 2.56 Hz, 1H); 7.13 (s, 1H); 7.55–7.62 (m, 3H); 7.69–7.76 (m, 2H); 8.17–8.24 (m, 2H); 12.85 (s, 1H). MS (ESI): 467.1 ($C_{24}H_{16}ClO_8$, $[M + H]^+$). Anal. Calcd for $C_{24}H_{15}ClO_8$: C, 61.75; H, 3.24%; Found: C, 61.82; H, 3.31%.

*4.3.3.18. 2-Bromo-6-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoic acid (**5r**).* White powder. Yield: 69%. M.p.: 183–184 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.86 (s, 3H); 5.32 (s, 2H); 6.79–6.86 (d, J = 2.43 Hz, 1H); 7.11 (s, 1H); 7.46–7.56 (m, 3H); 7.64–7.77 (m, 2H); 8.21–8.34 (m, 2H); 12.87(s, 1H). MS (ESI): 511.1 ($C_{24}H_{16}BrO_8$, $[M + H]^+$). Anal. Calcd for $C_{24}H_{15}BrO_8$: C, 56.38; H, 2.96%; Found: C, 56.42; H, 3.04%.

*4.3.3.19. 2-(2-(5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy) acetoxy)-6-methylbenzoic acid (**5s**).* White powder. Yield: 74%. M.p.: 179–181 °C ^{1}H NMR (300 MHz, d_6-DMSO): 2.37 (s, 3H); 3.87 (s, 3H); 5.34 (s, 2H); 6.73–6.82 (d, J = 2.54 Hz, 1H); 7.17 (s, 1H); 7.22–7.34 (m, 3H); 7.56–7.67 (m, 3H); 8.23–8.36 (m, 2H); 12.87 (s, 1H). MS (ESI): 447.1 ($C_{25}H_{19}O_8$, $[M + H]^+$). Anal. Calcd for $C_{25}H_{18}O_8$: C, 67.26; H, 4.06%; Found: C, 67.32; H, 4.12%.

*4.3.3.20. 2-Hydroxy-6-(2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetoxy)benzoic acid (**5t**).* White powder. Yield: 71%. M.p.: 210–211 °C ^{1}H NMR (300 MHz, d_6-DMSO): 3.86 (s, 3H); 5.33 (s, 2H); 6.83–6.92 (d, J = 2.74 Hz, 1H); 7.16 (s, 1H); 7.47–7.53 (m, 3H); 7.61–7.73 (m, 3H); 8.23–8.37 (m, 2H); 12.87 (s, 1H). MS (ESI): 449.1 ($C_{24}H_{17}O_9$, $[M + H]^+$). Anal. Calcd for $C_{24}H_{16}O_9$: C, 64.29; H, 3.60%; Found: C, 64.34; H, 3.71%.

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