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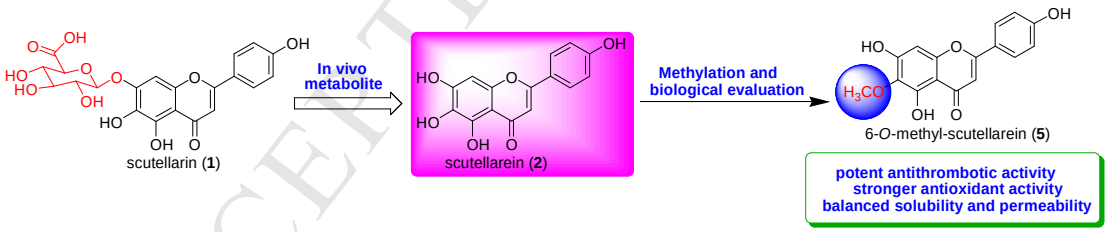
**ABSTRACT:** 6-*O*-methyl-scutellarein (**5**) demonstrated potent antithrombotic activity, stronger antioxidant activity and balanced solubility and permeability compared with scutellarin (**1**), which warrants further development for the treatment of ischemic cerebrovascular disease.



# Synthesis and biological evaluation of methylated scutellarein analogs based on metabolic mechanism of scutellarin *in vivo*

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**ABSTRACT:** Scutellarin (**1**) could be hydrolyzed into scutellarein (**2**) *in vivo* and then converted into methylated, sulfated and glucuronidated forms. In order to investigate the biological activities of these methylated metabolites, eight methylated analogs of scutellarein (**2**) were synthesized *via* semi-synthetic methods. The antithrombotic activities of these compounds were evaluated through the analyzation of prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB). Their antioxidant activities were assessed by measuring their scavenging capacities toward 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and the ability to protect PC12 cells against $H_2O_2$-induced cytotoxicity. Furthermore, the physicochemical properties of these compounds including aqueous solubility and lipophilicity were also investigated. The results showed that 6-*O*-methylscutellarein (**5**) demonstrated potent antithrombotic activity, stronger antioxidant activity and balanced solubility and permeability compared with scutellarin (**1**), which warrants further development of **5** as a promising lead for the treatment of ischemic cerebrovascular disease.

**Keywords:** Scutellarin, Scutellarein, Metabolism, Methylation, Thrombin, Antioxidant

## 1. Introduction

Nowadays, ischemic cerebrovascular disease is a common and frequently-occurring disease that seriously endangers human health, and it has been one of the leading causes of disability and death worldwide [1]. Some evidences suggest that thrombin plays a critical role in ischemic cerebrovascular disease [2], it is generated in response to vascular injury. As a multifunctional serine protease, thrombin can catalyze the proteolytic cleavage of the soluble plasma-protein fibrinogen to form insoluble fibrin, which leads to clot formation. In addition, thrombin also serves as a potent platelet agonist and amplifies its own generation by feedback activation of several steps in the coagulation cascade [3]. Furthermore, oxidative stresses are major causes of ischemic cerebrovascular disease [4,5], some reactive oxygen species (ROS) including superoxide anion radical ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide ($H_2O_2$), singlet oxygen ($^1O_2$) and nitric oxide ($NO^{\bullet}$) are constantly generated in the human body [6]. However, the excessive production of ROS may lead to increased level of low-density lipoprotein (LDL), and the oxidative modification of LDL can impair the endothelial derived relaxing factor (EDRF, nitric oxide, NO)-mediated bioactions [7].

On the above basis, some researchers have proposed and searched some natural products with antithrombotic capacity and antioxidant activity for the treatment of cerebrovascular disease [8]. Traditional Chinese medicines have been used clinically for many years and can be regarded as potential sources for drug discovery. Scutellarin (**1**) (Fig. 1), which is the main effective constituent (>85%) of breviscapine, a natural drug consisting of total flavonoids of Erigeron breviscapus (Vant.) Hand-Mazz. (Compositae), has been used for the treatment of cerebral infarction, angina pectoris and coronary heart disease with a large market share in China [9]. Nowadays, the research on scutellarin (**1**) has become a hot topic in China due to its distinguished efficacy in the clinical therapy. Pharmacological studies found that scutellarin (**1**) exhibited antithrombotic and antioxidant activities to attenuate neuronal damage, thus had a wide range of benefits to brain injury caused

by cerebral ischemia/reperfusion [10-12].

(**Fig. 1**)

Interestingly, some researches showed that scutellarin (**1**) is readily hydrolyzed into scutellarein (**2**) (Fig. 1) before absorption *in vivo*, and scutellarein (**2**) is absorbed into the blood and can metabolite into methylated, sulfated or glucuronidated forms [13]. 6-*O*-Methylscutellarein (**5**) (Fig. 1), which is one of the major *in vivo* metabolites, might be responsible for the therapeutic effects of scutellarin (**1**) [13]. In order to study the biological activities of 6-*O*-methylscutellarein (**5**) and other methylated scutellarein analogs, in this paper, we report here on the synthesis of a series of monomethylated scutellarein, dimethylated scutellarein and trimethylated scutellarein derivatives *via* semi-synthetic methods. Then the antithrombotic activities of these compounds were evaluated through the analyzation of prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB). Their antioxidant activities were assessed by measuring their scavenging capacities toward 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and the ability to protect PC12 cells against $H_2O_2$-induced cytotoxicity using MTT assay method. Furthermore, the physicochemical properties including aqueous solubility and lipophilicity were also investigated in this report.

## 2. Results and discussion

### 2.1. Synthesis

#### 2.1.1. Synthesis of monomethylated scutellarein analogs

The 4′-*O*-methylscutellarein (**3**) is synthesized as summarized in Scheme 1. Scutellarein (**2**) was obtained from scutellarin (**1**) by hydrolysis with 6N HCl in 90% ethanol under reflux [14,15]. Treatment of **2** with 1.5 equiv. of dichlorodiphenylmethane in diphenyl ether at 175 °C [16] led to the formation of desired product **11** in 85% yield after the reaction mixture had proceeded 30 min. Then the reaction of **11** with iodomethane (1.5 equiv.) afforded **12** in 91% yield with a methyl group at the C-4′ phenol hydroxyl position. Under hydrogenolysis

conditions, the diphenylmethylene ketal could be cleaved and the best results were obtained with 10% palladium on carbon as a catalyst in EtOH, this procedure allowed deprotection to afford **3** in 92% yield and no side product was detected by TLC analysis.

(**Scheme 1**)

The 5-*O*-methylscutellarein (**4**) is synthesized as summarized in Scheme 2. Reaction of **2** with an excess of chloromethyl methyl ether (4.5 equiv.) led to the formation of **13** in 72% yield, whose phenolic functions except the hydroxyl group at C-5 position were protected [17]. Because the phenol hydroxyl group at C-5 position could form a hydrogen bond with the carbonyl group at C-4 position, the reaction of **13** with iodomethane and $K_2CO_3$ did not work. Fortunately, the treatment of **13** with 1.5 equiv. of iodomethane and 2.0 equiv. of NaH in the solution of DMF led to **14** in 78% yield with a methyl group at the C-5 phenol hydroxyl position. At last, the hydrolysis of the MOM ether groups by hydrochloric acid provided the 5-*O*-methylscutellarein (**4**) in 90% yield.

(**Scheme 2**)

The synthesis of 6-*O*-methylscutellarein (**5**) is shown in Scheme 3. The reaction of **11** with 1.5 equiv. of benzyl bromide in the presence of $K_2CO_3$ afforded **15** in 93% yield. From compound **15**, we focused on the deprotection of diphenylmethylene ketal, two different methods were available for the cleavage of this protecting group: hydrogenolysis or hydrolysis. Fortunately, under hydrolysis conditions the diphenylmethylene ketal was deprotected by using the HOAc solution. This method gave **16** in 95% yield and no side product was detected by TLC analysis. Benzylation of **16** with benzyl bromide and $K_2CO_3$ in DMF led to the di-benzyl ether product **17** in 93% yield. A cross-peak observed in the ROESY spectrum of **17** between 5.03 ($-OCH_2Ph$) with 6.87 (C8–H) indicated that one of the two benzyl group was at C-7 phenol hydroxyl position, other cross-peaks between 5.23 ($-OCH_2Ph$) with 7.18 (C3′,5′–H) indicated that the other benzyl group was at C-4' phenol hydroxyl position. Treatment of **17** with 1.2 equiv. of iodomethane led selectively to **18** with the desired

methyl group was at the C-6 phenol hydroxyl position in 94% yield. Then the deprotection of di-benzyl groups under hydrogenation conditions using 10% palladium on carbon as the catalyst in THF/EtOH afforded **5** in 96% yield.

(**Scheme 3**)

Preparation of 7-*O*-methylscutellarein (**6**) is shown in Scheme 4. Scutellarein (**2**) was converted into **19** (90% yield) after it was reacted with acetic anhydride under the catalyst of 4-*N*,*N*-dimethylaminopyridine (DMAP) in pyridine. Interestingly, the reaction of **19** with benzyl bromide issued the substitution of acetic group by benzyl group to afford **20** in 70% yield. The position of benzyl group in **20** was confirmed by ROESY spectrum, a cross-peak between 5.36 ($-OCH_2$) with 6.87 (C8–H) indicated that the benzyl group was at the C-7 phenol hydroxyl position. This preferential reaction [18,19] might be that the electronically deficient C-4 carbonyl group in **19** was *para* to the C-7 acetoxy unit, thus the C-4 carbonyl group could accept electron density from the C-7 oxygen, which contributed to a weakening of the ester bond and facilitated its rupture, thus leading to the benzyl substituted intermediate **20**. Deprotection of the benzyl group in **20** was accomplished under hydrogenation conditions with 10% palladium on carbon as the catalyst in EtOH/$CH_2Cl_2$ gave **21** in 95% yield, then the treatment of **21** with iodomethane (1.5 equiv.) led to **22**. Finally, the hydrolysis of acetyl groups in **22** with a solution of sodium hydroxide afforded **6** in 81% yield.

(**Scheme 4**)

### 2.1.2. Synthesis of dimethylated and trimethylated scutellarein analogs

The synthesis of 6,7-dimethylscutellarein (**7**) is shown in Scheme 5. Treatment of **16** with 3.0 equiv. of iodomethane led to **23** in 86% yield, then the cleavage of the benzyl group by hydrogenolysis gave **7** in 91% yield. Because the reactivity of two phenol hydroxyl groups at C-4' and C-7 positions in 6-*O*-methylscutellarein (**5**) were very active, so the reaction of **5** with 1.5 equiv. of iodomethane produced **7** and **8** in 38% and 49% respectively (Scheme 5). Furthermore, the methylation of the free phenol hydroxyl function at C-6 and C-7 positions in **3** with iodomethane (3.5 equiv.)

afforded **9** and **10** in 32% and 54% respectively (Scheme 5).

(**Scheme 5**)

### 2.2. Biological investigations

#### 2.2.1. Antithrombotic activity

Because the antithrombotic activity can be assessed by measuring the prolongation of the plasma clotting time of TT, APTT, INR increasement of PT, and reduction of FIB content according to our previous studies [20], so the thrombin time of different compounds was investigated for TT, PT, APTT and FIB, and the results were shown in Table 1.

From the results we could see that the antithrombotic activity remained when the glucuronyl group in scutellarin (**1**) was hydrolyzed to produce scutellarein (**2**), although the PT decreased and the FIB content increased in scutellarein (**2**), the TT and APTT in scutellarein (**2**) increased compared to those in scutellarin (**1**), this result indicated that the glucuronyl group was not important for the antithrombotic activity. In the series of monomethylated scutellareins, the most active compounds were 6-*O*-methylscutellarein (**5**) and 7-*O*-methylscutellarein (**6**), these two compounds prolonged TT and PT, decreased FIB content compared to scutellarein (**2**). The less active compound was 4'-*O*-methylscutellarein (**3**), it decreased TT, APTT, and increased FIB content compared to **5**. Furthermore, three plasma coagulation parameters of **3** including TT, PT and APTT were decreased in the comparation to **6**. Interestingly, when the phenol hydroxyl group at C-5 position was etherized by methyl group, the antithrombotic activity decreased significantly, this result could be seen that the TT and APTT of 5-*O*-methylscutellarein (**4**) decreased, and the FIB content increased compared to those of 4'-*O*-methylscutellarein (**3**). So the phenol hydroxyl group at C-5 position was not methylated in the subsequent multiple-methly derivatives of scutellarein (**2**). These results showed that the methyl group etherized at the C-6 phenol hydroxyl position was very important to keep the antithrombotic activity.

In the series of dimethylated scutellarein analogs, the most active compound was 6,7-*O*-dimethylscutellarein (**7**), it prolonged TT and APTT, increased PT and decreased FIB content significantly compared to scutellarein (**2**). The less active compounds were 4',6-*O*-dimethylscutellarein (**8**) and 4',7-*O*-dimethylscutellarein (**9**), compound **8** only increased TT compared to **7**, compound **9** showed no stronger antithrombotic activity compared to **7** in the above four indicators. For compound 4',6,7-*O*-dimethylscutellarein (**10**), where the three phenol hydroxyl groups were all etherized by the methyl groups, the antithrombotic activity decreased, this phenomenon could be seen that **10** only increased TT compared to **7**. These pharmacological activity results indicated that the methyl group both etherized at the C-6 and C-7 phenol hydroxyl positions could improve the anticoagulant activity.

Interestingly, most of the synthesized analogs except **7** and **9** showed decreased APTT compared to **2** in the four indicators, these results indicated that the other three indicators including TT, PT and FIB might contribute to the antithrombic activity in these methylated scutellarein analogs.

(**Table 1**)

### 2.2.2. Antioxidant activity

The *in vitro* antioxidant activities [21] of these scutellarein derivatives were assessed by measuring their scavenging capacities toward DPPH assay and the ability to protect PC12 cells against $H_2O_2$-induced cytotoxicity.

#### 2.2.2.1. DPPH radical-scavenging activity

DPPH assay [22] measured the hydrogen-donating ability of antioxidants to convert the stable DPPH free radical into 1,1-diphenyl-2-picrylhydrazine [23]. The reaction was accompanied by a change in color from deep-yellow to light-yellow and was monitored spectrophotometrically.

The data obtained in the DPPH assay was depicted in Table 2. From the results we can infer that, the monomethylated scutellarein derivatives showed potent antioxidant activities. 6-*O*-methylscutellarein (**5**) ($IC_{50}$ = 23.67 μM), in which only the C-6 phenol hydroxy was etherized by the methyl group, showed stronger

antioxidant activity in this DPPH assay compared to scutellarin (**1**) ($IC_{50}$ = 27.93 μM). The less active compound was 4'-*O*-methylscutellarein (**3**), in which only the C-4' phenol hydroxy was etherized by the methyl group, it showed the $IC_{50}$ of 25.29 μM in the DPPH assay. 5-*O*-methylscutellarein (**4**), in which the C-5 phenol hydroxyl function was etherized by methyl group, showed less antioxidant activity compared to **3** with its $IC_{50}$ was 28.85 μM. 7-*O*-methylscutellarein (**6**), showed the least antioxidant activity in the series of monomethylated scutellarein analogs, with its $IC_{50}$ was 30.17 μM. Furthermore, if there were two methyl groups etherized at the phenol hydroxyl functions in scutellarein (**2**), the antioxidant activity decreased, these results could be seen that one methyl group etherized at the C-7 phenol hydroxyl position and C-4' phenol hydroxyl position in 6-*O*-methyl-scutellarein (**5**) to produce 6,7-*O*-dimethylscutellarein (**7**) and 4',6-*O*-dimethylscutellarein (**8**), their $IC_{50}$s changed into 189.88 μM and 169.82 μM respectively. Although 4'-*O*-methylscutellarein (**3**) and 7-*O*-methylscutellarein (**6**) showed strong antioxidant activity in this DPPH assay, when the methyl groups both etherized at the C-4' phenol hydroxyl and C-7 phenol hydroxyl positions to produce 4',7-*O*-dimethylscutellarein (**9**), the antioxidant activity was decreased with its $IC_{50}$ was 158.67 μM. At last, when the three phenol hydroxyl functions at C-4', C-6 and C-7 positions were all etherized by the methyl groups, the obtained product **10** lost its antioxidant activity with its $IC_{50}$ was 462.79 μM.

**2.2.2.2. Inhibitory Effect on PC12 Cells Induced by Oxidative Stress**

PC12 cells can adopt a neuronal phenotype and have been used extensively as a model for catecholamine-secreting neuronal cells [24]. Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which directly related to the number of living cells. So the neuroprotective effects of the synthesized methyl scutellarein derivatives were evaluated by the ability to protect PC12 cells against $H_2O_2$-induced cytotoxicity using MTT assay method [25].

The results of the inhibition rate against cell damage were summarized in Table

2. Comparison of the data reported in Table 2 indicated all the monomethylated scutellarein derivatives showed higher inhibitory effect than scutellarin (**1**) against $H_2O_2$-induced cytotoxicity in PC12 cells, especially for 4'-*O*-methylscutellarein (**3**) and 6-*O*-methyl-scutellarein (**5**). In a concentration of 50 μM, **3** and **5** showed 89.38% and 88.05% inhibition rate against cell damage, and in a concentration of 25 μM, **3** and **5** showed 63.27% and 62.39% inhibition rate against cell damage respectively, while the inhibiting rates of the parent compound **1** were 79.20% and 53.10% at 50 and 25 μM respectively. The less active compounds were 7-*O*-methylscutellarein (**6**) and 5-*O*-methylscutellarein (**4**), these two compounds exhibited 82.30% and 81.42% inhibition rate against cell damage in a concentration of 50 μM respectively. All the dimethylated scutellarein derivatives were less effective at protecting these neuronal cells against $H_2O_2$-induced oxidative injury than scutellarin (**1**), for example, 6,7-*O*-dimethylscutellarein (**7**) and 4',7-*O*-dimethylscutellarein (**9**) showed 76.11% and 74.34% inhibition rate against cell damage in a concentration of 50 μM respectively. Unfortunately, the trimethylated scutellarein derivative **10** lost its protective activity against cell damage with its inhibition rate was 20.35% in a concentration of 50 μM. In addition, all the synthesized analogs **3**-**10** showed a lower inhibitory effect than **2**, these results indicated that the presence of more phenol hydroxyl groups would be more important to protect the PC12 cells against $H_2O_2$-induced oxidative injury.

(**Table 2**)

### 2.2.3. Physicochemical property

Physicochemical profile plays a major role in modern drug discovery [26], aqueous solubility [27] and lipophilicity, usually expressed as n-octanol/water distribution coefficient, log*P* [28,29], are among the most important properties that can provide a guideline for predicting a drug's solubility characteristics [30]. These properties influence the distribution of drugs within the body since drugs must

possess some lipophilic properties to cross biological membranes, and hydrophilic properties to be taken up in systemic circulation.

**2.2.3.1. Water solubility determination**

The aqueous solubility of the synthesized methylated scutellarein derivatives was determined using UV spectrophotometer [31-33]. The results were shown in Table 3. Although the introduction of methyl group in the scutellarein could increase the lipid solubility, these methylated scutellarein derivatives still maintained some water solubility. Among the monomethylated scutellarein derivatives, 4'-*O*-methylscutellarein (**3**) and 5-*O*-methylscutellarein (**4**) showed better water solubility than the others with their values were 6.31 µg/ml and 5.72 µg/ml respectively. 7-*O*-methylscutellarein (**6**) and 6-*O*-methylscutellarein (**5**) exhibited good solubility in water with their values were 4.56 µg/ml and 4.27 µg/ml respectively. Introduction of more methyl groups decreased the water solubility of the scutellarein derivatives, this phenomenon could be seen that 4',7-*O*-dimethylscutellarein (**9**), 4',6-*O*-dimethylscutellarein (**8**) and 4',6,7-trimethylscutellarein (**10**) exhibited remarkable decreases in solubility with their values were 3.20 µg/ml, 1.36 µg/ml and 0.31 µg/ml respectively..

**2.2.3.2. Partition coefficient**

The partition coefficient between n-octanol and buffer for the methylated scutellarein derivatives compared to the parent compounds scutellarin (**1**) and scutellarein (**2**) was determined using traditional shake-flask method [34-36], the buffer included three different solutions which were 0.1mol/L HCl, 0.15mol/L NaCl and 0.15mol/L $NaHCO_3$, and the results were summerized in Table 3. The log*P* values of scutellarin (**1**) were found to be 0.5924, 0.3647 and -0.0513 in the three different solutions respectively. Introduction of methyl groups in scutellarein significantly altered the partition coefficient pattern in a range from 0.7846 to 2.6358 (Table 3). Interestingly, the 6-*O*-methylscutellarein (**5**) showed the best log*P* among the monomethylated scutellarein derivatives, with its values were 1.7651, 1.6805 and

1.0175 respectively. The log*P* values of 4',6,7-trimethylscutellarein (**10**) increased to 2.6358, 2.5492 and 2.2707 respectively when the three methyl groups etherized at the C-4', C-6 and C-7 phenol hydroxyl positions. These results showed that the introduction of the methyl group had a promising effect on the increasing of log*P* of the synthesized compounds compared to scutellarin (**1**), thus increased the lipophilicity of these compounds that may effect cell penetration.

(**Table 3**)

## 3. CONCLUSION

In summary, eight novel methylated scutellarein derivatives have been designed and synthesized based on the metabolic mechanism of scutellarin (**1**) *in vivo*. The antithrombotic activities of these compounds were evaluated through the analyzation of PT, APTT, TT and FIB, the results showed that one methyl group etherized at the C-6 phenol hydroxyl position was very important to keep the antithrombotic activity. Their *in vitro* antioxidant activity was evaluated by DPPH radical-scavenging activity assays and the protective effects on $H_2O_2$-induced cytotoxicity in PC12 cells using MTT assay method, the presence of the phenol hydroxyl group was very important to antioxidant activity. The results of aqueous solubility and lipophilicity showed that the introduction of the methyl group had a promising effect on the increasing of the lipophilicity of these compounds that may effect cell penetration. 6-*O*-methyl-scutellarein (**5**), which had only one methyl group etherized at the C-6 phenol hydroxyl position, demonstrated potent antithrombotic activity, stronger antioxidant activity and balanced solubility and permeability compared with scutellarin (**1**), warranted further development of this agent as a promising lead for the treatment of ischemic cerebrovascular disease.

## 4. Experimental

### 4.1. Chemical synthesis

Reagents and solvents were purchased from commercial sources and used

without further purification unless otherwise specified. Air- and moisture-sensitive liquids and solutions were transferred *via* syringe or stainless steel cannula. Organic solutions were concentrated by rotary evaporation below 45 ºC at approximately 20 mm Hg. All non-aqueous reactions were carried out under anhydrous conditions using flame-dried glassware within nitrogen atmosphere in dry, freshly distilled solvents, unless otherwise noted. Yields referred to chromatographically homogeneous materials, unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.15–0.20 mm Yantai silica gel plates (RSGF 254) using UV light as the visualizing agent. Chromatography was performed on Qingdao silica gel (160–200 mesh) using petroleum ether (60–90) and ethyl acetate as the eluanting solvent. $^1$H NMR spectra were obtained using a Bruker AV-300 (300 MHz) or Bruker AV-500 (500 MHz). Chemical shifts were recorded in ppm downfield from tetramethylsilane. *J* values were given in Hz. Abbreviations used were s (singlet), d (doublet), t (triplet), q (quartet), b (broad), and m (multiplet). ESI-MS spectra were recorded on a Waters Synapt HDMS spectrometer.

**4.1.1. 5,6,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (2)**

Water (10 ml) was added to a stirring mixture of scutellarin (**1**) (10.0 g, 21.6 mmol) and concentrated hydrochloric acid (120 ml) in ethanol (120 ml), then the reaction mixture was refluxed under an $N_2$ atmosphere for 36 h. After being cooled down to the room temperature, the mixture was poured into water, the solid obtained was filtered, and purified by column chromatography (50% ethyl acetate in petroleum ether) to afford **2** (1.05 g, 17.0% yield) as a yellow solid. $^1$H NMR (DMSO-$d_6$, 300 MHz) δ 6.57 (s, 1H, 8-H), 6.74 (s, 1H, 3-H), 6.91 (d, *J* = 8.8 Hz, 2H, 3',5'-H), 7.90 (d, *J* = 8.8 Hz, 2H, 2',6'-H), 8.71 (s, 1H, 6-OH), 10.29 (s, 1H, 7-OH), 10.44 (s, 1H, 4'-OH), 12.78 (s, 1H, 5-OH); ESI-MS: *m*/*z* 287 $[M+H]^+$.

**4.1.2.**

**9-Hydroxy-6-(4-hydroxyphenyl)-2,2-diphenyl-8H-[1,3]dioxolo[4,5-g]chromen-8-**

**one (11)**

To a stirring mixture of scutellarein (**2**) (10 g, 35 mmol) in diphenyl ether (200 ml) was added dichlorodiphenylmethane (18 g, 52.5 mmol, 1.5 equiv.), then the reaction mixture was heated at 175 °C for 30 min. After being cooled down to room temperature, petroleum ether (1000 ml) was added, the solid obtained was filtered, and purified by column chromatography (25% ethyl acetate in petroleum ether) to afford **11** (13.35 g, 85% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 6.69 (s, 1H, 3-H), 6.82 (s, 1H, 8-H), 6.96 (d, 2H, *J* = 8.7 Hz, 3′,5′-H), 7.47-7.50 (m, 6H, -Ph), 7.58-7.61 (m, 4H, -Ph), 7.94 (d, 2H, *J* = 8.7 Hz, 2′,6′-H), 10.41 (s, 1H, 4′-OH), 12.99 (s, 1H, 5-OH); ESI-MS: m/z 449 $[M–H]^-$.

**4.1.3.**

**9-Hydroxy-6-(4-methoxyphenyl)-2,2-diphenyl-8H-[1,3]dioxolo[4,5-g]chromen-8-one (12)**

To a stirring solution of **11** (200 mg, 0.44 mmol) in DMF (10 ml) was added $K_2CO_3$ (107 mg, 0.78 mmol, 1.75 equiv.) and iodomethane (0.041 ml, 0.66 mmol, 1.5 equiv.). After stirring at 0 °C for 2 h, the reaction mixture was allowed to warm to room temperature and the stirring was maintained for 6 h. Then the reaction mixture was partitioned between 100 ml ethyl acetate and 100 ml water, and the organic layer was washed with brine (100 ml), dried over $Na_2SO_4$, filtered and concentrated, the crude material was purified by column chromatography (33% ethyl acetate in petroleum ether) to afford **12** (186 mg, 91% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 3.88 (s, 3H, -$OCH_3$), 6.96 (s, 1H, 3-H), 7.11 (s, 1H, 8-H), 7.15 (d, 2H, *J* = 8.6 Hz, 3′,5′-H), 7.46-7.49 (m, 6H, -Ph), 7.55-7.60 (m, 4H, -Ph), 8.08 (d, 2H, *J* = 8.6 Hz, 2′,6′-H), 13.10 (s, 1H, 5-OH); ESI-MS: *m/z* 465 $[M+H]^+$

**4.1.4. 5,6,7-Trihydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one (3)**

To a solution of **12** (160 mg, 0.34 mmol) dissolved in ethanol (30 ml) was added 10% Pd/C (16 mg) with vigorous stirring. Then the reaction vessel was evacuated and

the atmosphere replaced with hydrogen. After 12 h, the reaction mixture was filtered, and the filtrate was concentrated, the crude material was purified by column chromatography (20% ethyl acetate in petroleum ether) to afford **3** (94mg, 92% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 3.91 (s, 3H, -$OCH_3$), 6.59 (s, 1H, 3-H), 6.78 (s, 1H, 8-H), 6.92 (d, 2H, *J* = 8.6 Hz, 3′,5′-H), 7.92 (d, 2H, *J* = 8.6 Hz, 2′,6′-H), 8.75 (s, 1H, 6-OH), 10.77 (s, 1H, 7-OH), 13.16 (s, 1H, 5-OH); ESI-MS: *m/z* 299 $[M–H]^-$.

**4.1.5.**

**5-Hydroxy-6,7-bis(methoxymethoxy)-2-(4-(methoxymethoxy)phenyl)-4H-chromen-4-one (13)**

Chloromethyl ether (2.39 ml, 31.5 mmol, 4.5 equiv.) was added to a stirring mixture of **2** (2 g, 7 mmol) and $K_2CO_3$ (5.80 g, 42 mmol, 6.0 equiv.) in dry acetone (100 ml) at room temperature. The reaction mixture was refluxed gently for 6 h. After being cooled down to room temperature, the reaction mixture was filtered, and the filtrate was concentrated, the crude material was purified by column chromatography (20% ethyl acetate in petroleum ether) to afford **13** (2.11 g, 72% yield) as a yellow solid. $^1$H NMR 300 MHz, DMSO-$d_6$) δ 3.66 (s, 3H, -$OCH_3$), 3.76(s, 3H, -$OCH_3$), 3.87 (s, 3H, -$OCH_3$), 5.35 (s, 2H, -$OCH_2$), 5.48 (s, 2H, -$OCH_2$), 5.60 (s, 2H, -$OCH_2$), 6.95 (s, 1H, 3-H), 7.09 (s, 1H, 8-H), 7.16 (d, 2H, *J* = 8.7 Hz, 3′,5′-H), 8.06 (d, 2H, *J* = 8.7 Hz, 2′,6′-H), 12.93 (s, 1H, 5-OH); ESI-MS: *m/z* 419 $[M+H]^+$.

**4.1.6.**

**5-Methoxy-6,7-bis(methoxymethoxy)-2-(4-(methoxymethoxy)phenyl)-4H-chromen-4-one (14)**

To a stirring solution of **13** (200 mg, 0.48 mmol) in DMF (10ml) at 0 ºC was added NaH (23 mg, 0.96 mmol, 2.0 equiv.), 30 min later, the iodomethane (0.04 ml, 0.66 mmol, 1.5 equiv.) was added, then the reaction mixture was allowed to warm to room temperature. After 6 h, the reaction mixture was partitioned between 100 ml ethyl

acetate and 100 ml water, and the organic layer was washed with brine (100 ml), dried over $Na_2SO_4$, filtered and concentrated, the crude material was purified by column chromatography (20% ethyl acetate in petroleum ether) to afford **14** (162 mg, 78% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 3.46 (s, 3H, -$OCH_3$), 3.47 (s, 3H, -$OCH_3$), 3.48 (s, 3H, -$OCH_3$), 3.49 (s, 3H, -$OCH_3$), 5.32 (s, 2H, -$OCH_2$), 5.33 (s, 2H, -$OCH_2$), 5.34 (s, 2H, -$OCH_2$), 6.43 (s, 1H, 3-H), 6.75 (s, 1H, 8-H), 7.23 (d, 2H, *J* = 8.7 Hz, 3′,5′-H), 8.06 (d, 2H, *J* = 8.7 Hz, 2′,6′-H); ESI-MS: *m/z* 433 $[M+H]^+$.

**4.1.7. 6,7-Dihydroxy-2-(4-hydroxyphenyl)-5-methoxy-4H-chromen-4-one (4)**

To a stirring solution of **14** (100 mg, 0.23 mmol) in ether (5ml) and dichloromethane (5ml) was added concentrated hydrochloric acid (1ml) at 0 ºC, then the reaction mixture was allowed to warm to room temperature. After 6 h, the resulting mixture was partitioned between 100 ml ethyl acetate and 100 ml water, and the organic layer was washed with brine (100 ml), dried over $Na_2SO_4$, filtered and concentrated, the crude material was purified by column chromatography (30% ethyl acetate in petroleum ether) to afford **4** (62 mg, 90% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 4.23 (s, 3H, -$OCH_3$), 6.67 (s, 1H, 3-H), 6.92 (d, 2H, *J* = 8.7 Hz, 3′,5′-H), 6.95 (s, 1H, 8-H), 7.92 (d, 2H, *J* = 8.7 Hz, 2′,6′-H), 8.73 (s, 1H, 6-OH), 10.30 (s, 1H, 4′-OH), 10.73 (s, 1H, 7-OH); ESI-MS: *m/z* 299 $[M–H]^-$.

**4.1.8. 6-(4-(Benzyloxy)phenyl)-9-hydroxy-2,2-diphenyl-8H-[1,3]dioxolo[4,5-g]chromen-8-one (15)**

To a stirring solution of **11** (200 mg, 0.44 mmol) in DMF (10 ml) was added $K_2CO_3$ (107 mg, 0.78 mmol, 1.75 equiv.) and benzyl bromide (0.078 ml, 0.66 mmol, 1.5 equiv.). After stirring at 0 °C for 2 h, the reaction mixture was allowed to warm to room temperature and the stirring was maintained for 12 h. Then the reaction mixture

was partitioned between 100 ml ethyl acetate and 100 ml water, and the organic layer was washed with brine (100 ml), dried over $Na_2SO_4$, filtered and concentrated, the crude material was purified by column chromatography (33% ethyl acetate in petroleum ether) to afford **15** (221 mg, 93% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 5.22 (s, 2H, -$OCH_2$), 6.95 (s, 1H, 3-H), 7.08 (s, 1H, 8-H), 7.22 (d, 2H, $J$ = 8.7 Hz, 3′,5′-H), 7.32-7.48 (m, 11H, -Ph), 7.55-7.61(m, 4H, -Ph), 8.04 (d, 2H, $J$ = 8.7 Hz, 2′,6′-H), 10.40 (s, 1H, 4′-OH), 13.10 (s, 1H, 5-OH); ESI-MS: *m/z* 541 $[M+H]^+$.

#### 4.1.9. 2-(4-(Benzyloxy)phenyl)-5,6,7-trihydroxy-4H-chromen-4-one (16)

Compound **15** (150 mg, 0.28 mmol) was dissolved in 20 ml of HOAc/$H_2O$ (4:1) solution and the reaction mixture was refluxed under a $N_2$ atmosphere for 1.5 h. After being cooled down to the room temperature, the mixture was poured into 100 ml water, extracted with ethyl acetate (50 ml×3), then the organic layer was washed with brine (100 ml), dried over $Na_2SO_4$, filtered and concentrated, the crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to afford **16** (100 mg, 95% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 5.28 (s, 2H, -$OCH_2$), 6.81 (s, 1H, 3-H), 6.92 (d, 2H, $J$ = 8.7 Hz, 3′,5′-H), 7.00 (s, 1H, 8-H), 7.35-7.45 (m, 3H, -Ph), 7.51-7.54 (m, 2H, -Ph), 7.94 (d, 2H, $J$ = 8.7 Hz, 2′,6′-H), 8.74 (s, 1H, 6-OH), 10.35 (s, 1H, 7-OH), 12.69 (s, 1H, 5-OH); ESI-MS: *m/z* 375 $[M–H]^-$.

#### 4.1.10. 7-(Benzyloxy)-2-(4-(benzyloxy)phenyl)-5,6-dihydroxy-4H-chromen-4-one (17)

To a stirring solution of **16** (200 mg, 0.53 mmol) in dry DMF (20 ml) was added $K_2CO_3$ (109 mg, 0.80 mmol, 1.5 equiv.) and benzyl bromide (0.08 ml, 0.68 mmol, 1.3 equiv.) at 0 °C, then the mixture was warmed to room temperature. After 12 h, the reaction mixture was partitioned between 100 ml ethyl acetate and 100 ml water, then the ethyl acetate layer was washed with brine (100 ml), dried over $Na_2SO_4$, filtered and concentrated, the crude material was purified by column chromatography (25%

ethyl acetate in petroleum ether) to afford **17** (230 mg, 93% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 5.03 (s, 2H, -$OCH_2$), 5.23 (s, 2H, -$OCH_2$), 6.62 (s, 1H, 3-H), 6.87 (s, 1H, 8-H), 7.18 (d, 2H, *J* = 8.6 Hz, 3′,5′-H), 7.31-7.53 (m, 10H, -Ph), 8.04 (d, 2H, *J* = 8.6 Hz, 2′,6′-H), 10.82 (s, 1H, 6-OH), 13.11 (s, 1H, 5-OH); ESI-MS: *m/z* 465 $[M–H]^-$.

**4.1.11.**

**7-(Benzyloxy)-2-(4-(benzyloxy)phenyl)-5-hydroxy-6-methoxy-4H-chromen-4-one (18)**

To a stirring solution of **17** (84 mg, 0.18 mmol) in dry DMF (20 ml) was added $K_2CO_3$ (48 mg, 0.35 mmol, 1.4 equiv.) and iodomethane (0.014 ml, 0.22 mmol, 1.2 equiv.) at room temperature. After 12 h, the reaction mixture was then partitioned between 100 ml ethyl acetate and 100 ml water. The ethyl acetate layer was then washed with brine (100 ml), dried over $Na_2SO_4$, filtered and concentrated, the crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to afford **18** (81 mg, 94% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 4.00 (s, 3H, -$OCH_3$), 4.95 (s, 2H, -$OCH_2$), 5.28 (s, 2H, -$OCH_2$), 6.62 (s, 1H, 3-H), 6.94 (d, 2H, *J* = 8.6 Hz, 3′,5′-H), 7.00 (s, 1H, 8-H), 7.32-7.42 (m, 6H, -Ph), 7.44-7.54 (m, 4H, -Ph), 7.94 (d, 2H, *J* = 8.6 Hz, 2′,6′-H), 12.69 (s, 1H, 5-OH); ESI-MS: *m/z* 481 $[M+H]^+$.

**4.1.12. 5,7-Dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4H-chromen-4-one (5)**

To a solution of **18** (100 mg, 0.21 mmol) dissolved in ethanol (10 ml) and THF (10 ml) was added 10% Pd/C (2 mg) with vigorous stirring. The reaction vessel was then evacuated and the atmosphere replaced with hydrogen. After 8 h, the reaction mixture was filtered and the filtrate was concentrated, the crude material was purified by column chromatography (50% ethyl acetate in petroleum ether) to afford **5** (60 mg, 96% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 3.72 (s, 3H, -$OCH_3$), 6.58 (s, 1H, 3-H), 6.89 (d, 2H, *J* = 8.6 Hz, 3′,5′-H), 7.11 (s, 1H, 8-H), 7.87 (d, 2H, *J* =

8.6 Hz, 2′,6′-H), 10.19 (s, 1H, 4′-OH), 10.72 (s, 1H, 7-OH), 12.76 (s, 1H, 5-OH); ESI-MS: *m/z* 299 $[M–H]^-$.

**4.1.13. 5,6,7-Triacetoxy-2-(4-acetoxyphenyl)-4H-chromen-4-one (19)**

Pyridine (5 ml) and 4-dimethylaminopyridine (DMAP) (12.2 mg, 0.1 mmol) were added to a stirring solution of **2** (286 mg, 1.0 mmol) in acetic anhydride (5 ml) at 0 °C, then the mixture was warmed to room temperature. After 4 h, the reaction mixture was poured into 100 ml water, and the solid obtained was filtered and purified by column chromatography (25% ethyl acetate in petroleum ether) to afford **19** (408 mg, 90% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 2.32 (s, 3H, –$COCH_3$), 2.35 (s, 3H, –$COCH_3$), 2.37 (s, 3H, –$COCH_3$), 2.38 (s, 3H, –$COCH_3$), 6.95 (s, 1H, 8-H), 7.36 (d, *J* = 8.7 Hz, 2H, 3',5'-H), 7.84 (s, 1H, 3-H), 8.14 (d, *J* = 8.7 Hz, 2H, 2',6'-H); ESI-MS: *m/z* 455 $[M+H]^+$.

**4.1.14. 7-(Benzyloxy)-5,6-diacetoxy-2-(4-acetoxyphenyl)-4H-chromen-4-one (20)**

To a stirring solution of **19** (227 mg, 0.50 mmol) in dry acetone (20 ml) was added $K_2CO_3$ (484 mg, 3.50 mmol, 7.0 equiv.) and benzyl bromide (0.07 ml, 0.60 mmol, 1.2 equiv.), after the reaction was refluxed for 6 h, the mixture was partitioned between 100 ml ethyl acetate and 100 ml water. The ethyl acetate layer was then washed with brine (100 ml), dried over $Na_2SO_4$, filtered, and concentrated, the crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to afford **20** (186 mg, 74% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 2.31 (s, 3H, –$COCH_3$), 2.32 (s, 3H, –$COCH_3$), 2.34 (s, 3H, –$COCH_3$), 5.36 (s, 2H, $CH_2$), 6.87 (s, 1H, 8-H), 7.36–7.45 (m, 7H, 3',5'-H and ArH), 7.60 (s, 1H, 3-H), 8.13 (d, *J* = 8.8 Hz, 2H, 2',6'-H); ESI-MS: *m/z* 503 $[M+H]^+$.

**4.1.15. 7-Hydroxy-5,6-diacetoxy-2-(4'-acetoxyphenyl) -4H-1-benzopyran-4-one (21)**

To a stirring mixture of **20** (426 mg, 0.85 mmol) in dichloromethane (50 ml) and

ethanol (50 ml) was added 10% Pd/C (43 mg). The reaction vessel was then evacuated and the atmosphere replaced with hydrogen. After 12 h, the reaction mixture was filtered, concentrated and purified by column chromatography (25% ethyl acetate in petroleum ether) to afford **21** (322 mg, 92% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 2.31 (s, 3H, -OC-$CH_3$), 2.34 (s, 3H, -OC-$CH_3$), 2.35 (s, 3H, -OC-$CH_3$), 6.78 (s, 1H, 3-H), 7.08 (s, 1H, 8-H), 7.38 (d, 2H, *J* = 8.8Hz, 3′,5′-H), 8.18 (d, 2H, *J* = 8.8Hz, 2′,6′-H), 11.52 (s, 1H, 7-OH); ESI-MS: *m/z* 411 $[M–H]^-$.

### 4.1.16. 7-Methoxy-5,6-diacetoxy-2-(4'-acetoxyphenyl) -4H-1-benzopyran-4-one (22)

To a stirring solution of **21** (200 mg, 0.49 mmol) in dry DMF (10 ml) was added $K_2CO_3$ (117 mg, 0.85 mmol, 1.75 equiv.) and iodomethane (0.05 ml, 0.74 mmol, 1.5 equiv.) at room temperature. After 6 h, the reaction mixture was then partitioned between 100 ml ethyl acetate and 100 ml water. The ethyl acetate layer was then washed with brine (100 ml), dried over $Na_2SO_4$, filtered and concentrated, the crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to afford **22** (188 mg, 90% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 2.30 (s, 3H, -OC-$CH_3$), 2.31 (s, 3H, -OC-$CH_3$), 2.33 (s, 3H, -OC-$CH_3$), 4.09 (s, 3H, -$CH_3$), 6.88 (s, 1H, 3-H), 7.36 (d, 2H, *J* = 8.8Hz, 3′,5′-H), 7.38 (s, 1H, 8-H), 8.12 (d, 2H, *J* = 8.8Hz, 2′,6′-H); ESI-MS: *m/z* 427$[M+H]^+$。

### 4.1.17. 5,6-Dihydroxy-2-(4-hydroxyphenyl)-7-methoxy-4H-chromen-4-one (6)

A solution of sodium hydroxide (0.5 M, 1 ml) was added to a stirring mixture of **22** (85 mg, 0.20 mmol) in methanol (10 ml) at 0 °C under $N_2$ atmosphere, after 1h, the mixture was then partitioned between 100 ml ethyl acetate and 100 ml water. The ethyl acetate layer was then washed with brine (100 ml), dried over $Na_2SO_4$, filtered, and concentrated, the crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to afford **6** (49 mg, 81% yield) as a yellow solid. $^1$H

NMR (300 MHz, DMSO-$d_6$) δ 4.49 (s, 3H, -$CH_3$), 6.59 (s, 1H, 3-H), 6.78 (s, 1H, 8-H), 6.93 (d, 2H, *J* = 8.8Hz, 3′,5′-H), 7.92 (d, 2H, *J* = 8.8Hz, 2′,6′-H), 10.33 (s, 1H, 4′-OH), 13.16 (s, 1H, 5-OH); ESI-MS: *m/z* 299 $[M–H]^-$.

#### 4.1.18. 2-(4-(Benzyloxy)phenyl)-5-hydroxy-6,7-dimethoxy-4H-chromen-4-one (23)

To a stirring solution of **16** (200 mg, 0.53 mmol) in dry DMF (10 ml) was added $K_2CO_3$ (256 mg, 1.86 mmol, 3.5 equiv.) and iodomethane (0.11 ml, 1.59 mmol, 3.0 equiv.) at room temperature. After 6 h, the reaction mixture was then partitioned between 100 ml ethyl acetate and 100 ml water. The ethyl acetate layer was then washed with brine (100 ml), dried over $Na_2SO_4$, filtered and concentrated, the crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to afford **23** (184 mg, 86% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 3.76 (s, 3H, -$CH_3$), 3.87 (s, 3H, -$CH_3$), 5.29 (s, 2H, -$CH_2$), 6.95 (s, 1H, 3-H), 7.08 (s, 1H, 8-H), 7.14 (d, 2H, *J* = 8.7 Hz, 3′,5′-H), 7.35-7.53 (m, 8H, -Ph), 7.65-7.74 (m, 2H, -Ph), 8.06 (d, 2H, *J* = 8.7 Hz, 2′,6′-H), 12.90 (s, 1H, 5-OH); ESI-MS: *m/z* 405 $[M+H]^+$.

#### 4.1.19. 5-Hydroxy-2-(4-hydroxyphenyl)-6,7-dimethoxy-4H-chromen-4-one (7)

To a solution of **23** (100 mg, 0.25 mmol) dissolved in ethanol (30 ml) was added 10% Pd/C (10 mg) with vigorous stirring. The reaction vessel was then evacuated and the atmosphere replaced with hydrogen. After 12 h, the reaction mixture was filtered and the filtrate concentrated, the crude material was purified by column chromatography (20% ethyl acetate in petroleum ether) to afford **7** (71 mg, 91% yield) as a yellow solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 3.92 (s, 3H, -$CH_3$), 3.98 (s, 3H, -$CH_3$), 6.86 (s, 1H, 3-H), 7.52 (s, 1H, 8-H), 7.36 (d, 2H, *J* = 8.7 Hz, 3′,5′-H), 8.15 (d, 2H, *J* = 8.7 Hz, 2′,6′-H), 10.40 (s, 1H, 4′-OH), 13.05 (s, 1H, 5-OH); ESI-MS: *m/z* 313 $[M–H]^-$.

**4.1.20. 5,7-Dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one (8)**

To a stirring solution of **5** (200 mg, 0.67 mmol) in dry DMF (10 ml) was added $K_2CO_3$ (184 mg, 1.33 mmol, 2.0 equiv.) and iodomethane (0.07 ml, 1.00 mmol, 1.5 equiv.) at room temperature. After 6 h, the reaction mixture was then partitioned between 100 ml ethyl acetate and 100 ml water. The ethyl acetate layer was then washed with brine (100 ml), dried over $Na_2SO_4$, filtered and concentrated, the crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to afford **7** (80 mg, 38% yield) and **8** (103mg, 49% yield) as yellow solids. **8** $^1$H NMR (300 MHz, DMSO-$d_6$) δ 3.40 (s, 3H, -$CH_3$), 3.51 (s, 3H, -$CH_3$), 6.63 (s, 1H, 3-H), 6.88 (s, 1H, 8-H), 7.19 (d, 2H, *J* = 8.7 Hz, 3′,5′-H), 8.04 (d, 2H, *J* = 8.7 Hz, 2′,6′-H), 10.91 (s, 1H, 7-OH), 13.07 (s, 1H, 5-OH); ESI-MS: *m/z* 313 $[M–H]^-$.

**4.1.21. Synthesis of 9 and 10**

To a stirring solution of **3** (200 mg, 0.67 mmol) in dry DMF (10 ml) was added $K_2CO_3$ (368 mg, 2.67 mmol, 4.0 equiv.) and iodomethane (0.16 ml, 2.33 mmol, 3.5 equiv.) at room temperature. After 8 h, the reaction mixture was then partitioned between 100 ml ethyl acetate and 100 ml water. The ethyl acetate layer was washed with brine (100 ml), dried over $Na_2SO_4$, filtered and concentrated, the crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to afford **9** (67 mg, 32% yield) and **10** (118 mg, 54% yield) as yellow solids

**4.1.21.1 5,6-Dihydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one (9)**

$^1$H NMR (300 MHz, DMSO-$d_6$) δ 3.73 (s, 6H, -$CH_3$), 3.91 (s, 6H, -$CH_3$), 6.67 (s, 1H, 3-H), 7.14 (s, 1H, 8-H), 7.16 (d, 2H, *J* = 8.7 Hz, 3′,5′-H), 7.98 (d, 2H, *J* = 8.6 Hz, 2′,6′-H), 8.98 (s, 1H, 6-OH), 12.59 (s, 1H, 5-OH); ESI-MS: *m/z* 313 $[M–H]^-$.

**4.1.21.2 5-Hydroxy-6,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one (10)**

$^1$H NMR (300 MHz, DMSO-$d_6$) δ 3.78 (s, 3H, -$CH_3$), 3.91 (s, 3H, -$CH_3$), 3.94 (s, 3H, -$CH_3$), 6.71 (s, 1H, 3-H), 7.16 (d, 2H, *J* = 8.6 Hz, 3′,5′-H), 7.21 (s, 1H, 8-H), 8.06 (d, 2H, *J* = 8.7 Hz, 2′,6′-H), 12.95 (s, 1H, 5-OH); ESI-MS: *m/z* 329 $[M+H]^+$。

**4.2. Antithrombotic assay**

#### 4.2.1. Blood collection

Male New Zealand white rabbits, weighing 2-2.5 kg were obtained from the experimental animal center of Nanjing University of Chinese Medicine and were approved by Animal Ethics Committee of Nanjing University of Chinese Medicine. They were kept in plastic cages at 22 ± 2°C with free access to pellet food and water and on a 12 h light/dark cycle. Animal welfare and experimental procedures were carried out in accordance with the guide for the care and use of laboratory animals (National Research Council of USA, 1996) and related ethical regulations of Nanjing University of Chinese Medicine. Rabbits were anesthetized with pentobarbital (50 mg/kg) and blood was drawn from the common carotid artery. Blood was collected into plastic tubes with 3.8% sodium citrate (citrate/blood: 1/9, v/v) for plasma anticoagulation. Platelet-poor plasma (PPP) was separated from blood by centrifugation at 3000 rpm for 10 min.

#### 4.2.2. Plasma antithrombotic assay

TT, PT, APTT and FIB were examined with commercial kits following the manufacturer's instructions by a coagulometer (Model LG-PABER-I, Steellex Co., China). All the compounds were dissolved in 80% ethanol, and the concentration was 100 μM. TT was determined by incubating 40 μl PPP solution for 3 min at 37 °C, followed by addition of 40 μl thrombin solution and 20 μl sample for 3 min at 37 °C. PT was determined by incubating 40 μl PPP solution for 3 min at 37 °C, followed by addition of 40 μl thromboplastin agent and 20 μl sample. APTT was determined by incubating 10 μl sample solution and 50 μl PPP solution with 50 μl APTT-activating agent for 3 min at 37 °C, followed by addition of 50 μl $CaCl_2$. FIB was determined by incubating 10 μl PPP solution with 90 μl imidazole buffer for 3 min at 37 °C, followed by addition of 50 μl FIB agent and 10 μl sample solution. The antithrombotic activity was assessed by assaying the prolongation of the plasma clotting time of TT, APTT, INR increasement of PT, and reduction of FIB content.

### 4.3. Antioxidant assay

#### 4.3.1. DPPH radical scavenging assay

The DPPH radical scavenging activity was measured according to previous studies [14]. The solution of the sample (100 μL) in dimethylsulfoxide (DMSO) was added to 100 μL of DPPH radical in ethanol (0.2 mM) in 96-well plate. The sample solution refers to the tested compounds and the reference antioxidants at various concentrations, as well as DMSO as a control. The solutions of the tested compounds had concentrations ranging from 5 μM to 500 μM. The reaction leading to the scavenging of DPPH radical was completed within 30 min at 25 ºC. The absorbance of the mixture was then measured at 517 nm with a microplate reader. The inhibition of DPPH radical was expressed as percentage [14]: Scavenging rate % = $[1 - (A_1 - A_2)/A_0] \times 100\%$, where $A_1$ was the absorbance of the test substance acted with DPPH, $A_2$ was the absorbance of the test substance without DPPH and $A_0$ was the absorbance of the blank. The $IC_{50}$ value was defined as the concentration of sample that causes 50% loss of the DPPH radical.

**4.3.2. PC12 cells antioxidant assay**

Cell viability was estimated by MTT assay. Briefly, PC12 cells were seeded in 96-well plates at a density of $5\times10^4$ cells/ml (100 μl per well), then they were cultured at 37 °C in humidified air containing 5% $CO_2$ for 24 h. The experiment was divided into control group and $H_2O_2$ damage model group, each set five wells. In the control group was serum-free DMEM medium. The $H_2O_2$ damage model group was exposure to 400 μM $H_2O_2$ for 1 h before the test drugs were added in. Drug concentrations were 50 μM and 25 μM. The cells were incubated with 20 μl of MTT solution for 4 h at 37 °C. The formazan crystals formed in intact cells were dissolved in 150 μl of DMSO, and then vigorously shaking for 10 min. Finally, the absorbance was assessed at 517 nm. The inhibiting rate of $H_2O_2$-induced cytotoxicity in PC12 cells was calculated as inhibiting rate (%) = $[(A_1 - A_2)/(A_0 - A_2)] \times 100\%$, where $A_1$ was the absorbance in the presence of sample and $H_2O_2$, $A_2$ was the absorbance in the presence of $H_2O_2$ (model), $A_0$ was the absorbance without sample and $H_2O_2$ (normal).

### 4.4. Physicochemical properties

#### 4.4.1. Solubility determination

The solubility of scutellarein derivatives in water was determined using the known method [31-33] with minor modifications. The UV/UV device is composed of a UV source (for UV photodegradation) and a UV absorption detector (for on-line UV measurement). The UV source is a low pressure mercury lamp and the UV detector is a UV-vis spectrophotometer Anthelie (Secomam) controlled by software Dathelie, version 4.1f. The pathlength of the Suprasil quartz cell is 10 mm and the scan speed is 2000 nm $min^{-1}$. Under the influence of UV radiation, methylated scutellarein derivatives were monitored by UV absorption spectrophotometry at the wavelength of maximum absorbance (334 nm) from the whole spectrum using a multicomponent exploitation method. Each tested compound (250 μg) was dissolved in 25 ml $CH_3OH$. The solutions of the tested compounds had concentrations ranging from 0.1 μg/ml to 10 μg/ml. Different concentration solutions of each compound were determined by UV scanning, and the absorbances were obtained. The results showed a good linear relationship, then all the standard curves were completed. Each tested compound (150 μg) was ultrasound dissolved in 10 ml pure water for 1 h at room temperature. The solutions were stranded for 30 min and centrifuged at the speed of 30000 r/min. The aqueous solution of each compound was determined by UV scanning, and the absorbances were obtained. Then through the analyzation of standard curve, all the compounds in the water solubility were obtained.

#### 4.4.2. Determination of partition coefficient

The partition coefficient (log*P*) between n-octanol and the buffer was determined by a slight modification of the previous method [36]. Briefly, 100 μl of a stock solution (10 μg/ml) was diluted with 1.9 ml of appropriate buffer solution (0.1mol/L HCl, 0.15mol/L NaCl and 0.15mol/L $NaHCO_3$ respectively) and mixed with 2 ml of n-octanol (the organic and aqueous phase was mutually saturated), the vials were protected from light by wrapping in aluminum foil. The two phases were

vortexed for 3 min and agitated for 5 h in a shaking water bath at 25 ± 0.1 ºC. After equilibration, the n-octanol phase was removed with a Pasteur pipette and both phases were assayed spectrophotometrically ($\lambda_{max}$ = 334 nm) to determine drug concentration. The partition coefficient was calculated as the ratio between molar concentration in n-octanol and aqueous phase. The total concentration in both phases was measured by spectrophotometry and the experimental partition coefficients (log*P*) was calculated by the following equation: log*P* = log[C] n-octanol/[C] aqueous.

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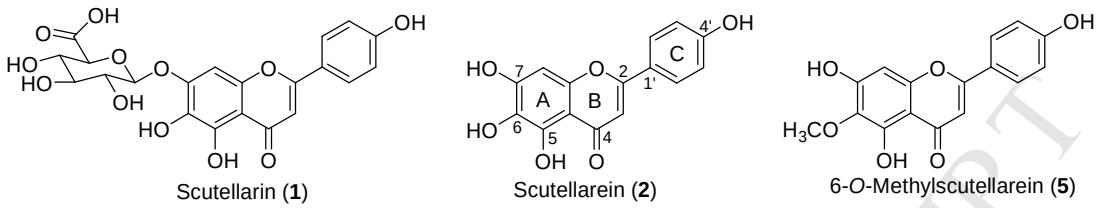
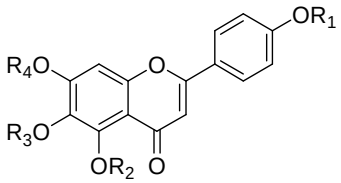



**Fig. 1.** Chemical structures of scutellarin (**1**), scutellarein (**2**) and 6-*O*-methylscutellarein (**5**).

**Table 1** Effect of *O*-methylated analogs of scutellarein on thrombin time.




| Compd. (100 μM) | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Plasma coagulation parameters | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | TT (s) | PT (s) | APTT (s) | FIB (g/l) |
| **Control** | — | — | — | — | 20.25±1.02 | 4.99±0.18 | 30.58±1.55 | 7.02±0.16 |
| **1** | H | H | H | glucuronyl | 23.08±0.87 | 6.28±0.08 | 34.18±2.25 | 6.13±0.15 |
| **2** | H | H | H | H | 24.40±1.59 | 5.93±1.21 | 36.65±3.60 | 6.84±0.10 |
| **3** | $CH_3$ | H | H | H | 24.98±0.66 | 6.40±0.50 | 31.53±3.67 | 5.67±0.19 |
| **4** | H | $CH_3$ | H | H | 22.60±1.32 | 6.65±0.15 | 30.53±0.70 | 5.58±0.05 |
| **5** | H | H | $CH_3$ | H | 25.96±1.16 | 6.25±0.05 | 33.10±1.68 | 5.38±0.11 |
| **6** | H | H | H | $CH_3$ | 25.48±4.09 | 6.55±0.25 | 34.49±1.94 | 5.74±0.26 |
| **7** | H | H | $CH_3$ | $CH_3$ | 25.10±0.78 | 6.95±0.04 | 39.42±1.69 | 5.65±0.18 |
| **8** | $CH_3$ | H | $CH_3$ | H | 27.45±3.98 | 6.53±0.32 | 30.48±0.48 | 5.92±0.14 |
| **9** | $CH_3$ | H | H | $CH_3$ | 23.33±1.10 | 6.93±0.24 | 38.28±4.34 | 5.94±0.10 |
| **10** | $CH_3$ | H | $CH_3$ | $CH_3$ | 29.03±4.51 | 6.75±0.21 | 36.00±1.61 | 5.96±0.13 |

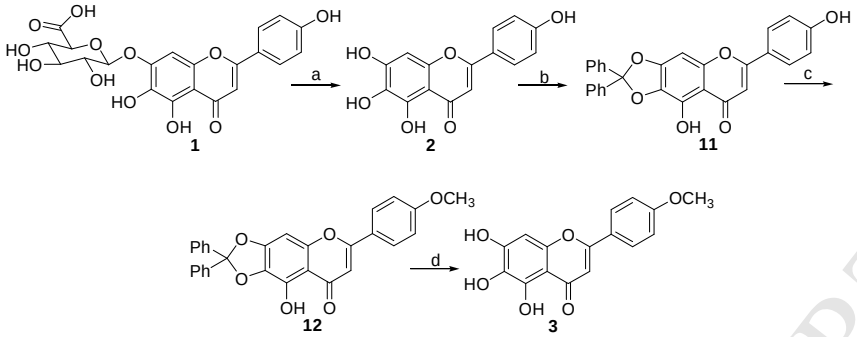
Data represent mean ± S.D. $n = 4$.

**Table 2** In vitro antioxidatant activity in DPPH assay and protective effect against hydrogen peroxide-induced cytotoxicity in PC12 cells.

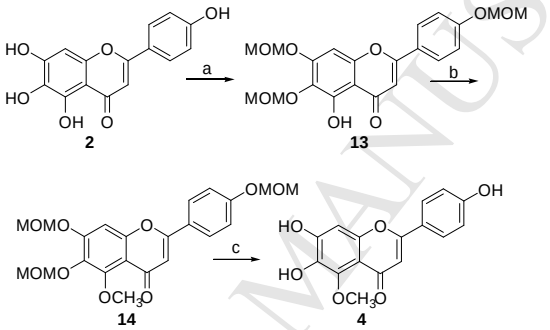
| Compd. | DPPH assay $IC_{50}$ (μM) | PC12 cells assay | | |
|---|---|---|---|---|
| | | Concentrations of compd. (μM) | $A_{517}$ | Inhibiting rate (%) |
| **Normal** | — | — | 0.568 ± 0.005 | — |
| **Model** | — | — | 0.342 ± 0.004 | — |
| **1** | 27.93 | 50 | 0.521 ± 0.003 | 79.20 |
| | | 25 | 0.462 ± 0.002 | 53.10 |
| **2** | 22.58 | 50 | 0.548 ± 0.004 | 91.15 |
| | | 25 | 0.488 ± 0.003 | 64.60 |
| **3** | 25.29 | 50 | 0.544 ± 0.005 | 89.38 |
| | | 25 | 0.485 ± 0.003 | 63.27 |
| **4** | 28.85 | 50 | 0.526 ± 0.003 | 81.42 |
| | | 25 | 0.465 ± 0.002 | 54.42 |
| **5** | 23.67 | 50 | 0.541 ± 0.004 | 88.05 |
| | | 25 | 0.483 ± 0.005 | 62.39 |
| **6** | 30.17 | 50 | 0.528 ± 0.003 | 82.30 |
| | | 25 | 0.469 ± 0.006 | 56.19 |
| **7** | 189.88 | 50 | 0.514 ± 0.004 | 76.11 |
| | | 25 | 0.460 ± 0.003 | 52.21 |
| **8** | 169.82 | 50 | 0.496 ± 0.005 | 68.14 |
| | | 25 | 0.442 ± 0.002 | 44.25 |
| **9** | 158.67 | 50 | 0.510 ± 0.004 | 74.34 |
| | | 25 | 0.451 ± 0.003 | 48.23 |
| **10** | 462.79 | 50 | 0.388 ± 0.004 | 20.35 |
| | | 25 | 0.367 ± 0.005 | 11.06 |

**Table 3.** The water solubility and partition coefficient of scutellarein methyl derivatives.

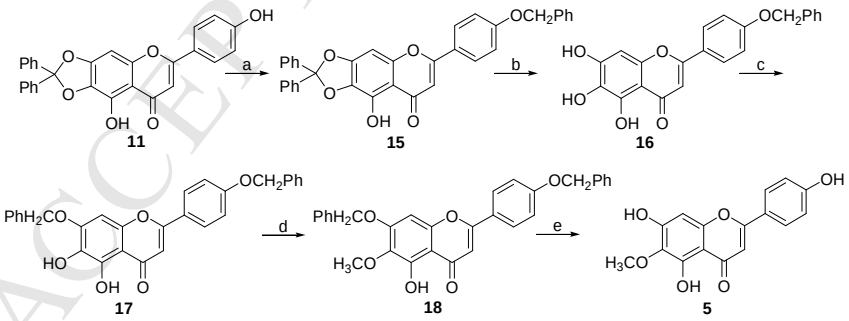
| Comp. | Solubility (μg/ml) | lg*P* | | |
|---|---|---|---|---|
| | | n-octanol/0.1 M HCl | n-octanol/0.15M NaCl | n-octanol/0.15 M $NaHCO_3$ |
| **1** | 7.62 | 0.5924 | 0.3647 | -0.0513 |
| **2** | 6.85 | 0.8916 | 0.7720 | 0.4799 |
| **3** | 6.31 | 1.6458 | 1.4092 | 0.7846 |
| **4** | 5.72 | 1.5550 | 1.4684 | 0.8923 |
| **5** | 4.27 | 1.7651 | 1.6805 | 1.0175 |
| **6** | 4.56 | 1.5928 | 1.5374 | 1.0343 |
| **7** | 1.53 | 2.1942 | 2.1354 | 1.8086 |
| **8** | 1.36 | 2.1402 | 2.0937 | 1.8631 |
| **9** | 3.20 | 1.9372 | 1.7873 | 1.3959 |
| **10** | 0.31 | 2.6358 | 2.5492 | 2.2707 |



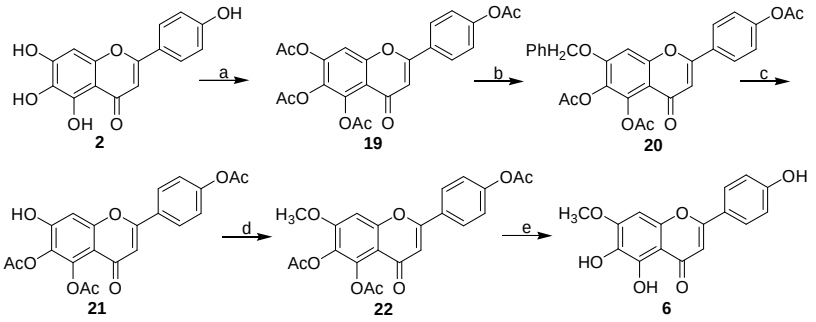
**Scheme 1.** Reagents and conditions: (a) HCl, EtOH, $N_2$, reflux, 36 h, 17%; (b) $Ph_2CCl_2$ (1.5 equiv.), $Ph_2O$, 175°C, 30min, 85%; (c) $CH_3I$ (1.5 equiv.), $K_2CO_3$ (1.75 equiv.), DMF, 25ºC, 6h, 91%; (d) $H_2$ (1 atm), Pd/C (10 wt%), EtOH, 25ºC, 12h, 92%.



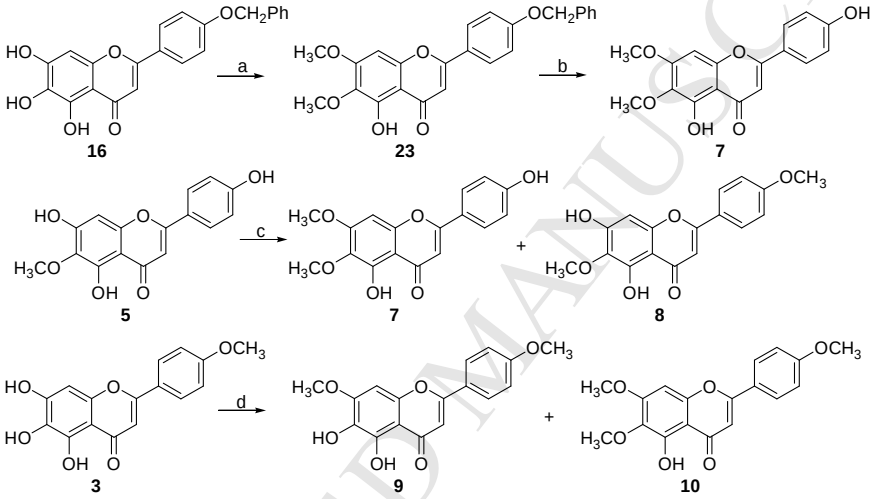
**Scheme 2.** Reagents and conditions: (a) MOMCl (4.5 equiv.), $K_2CO_3$ (6 equiv.), acetone, reflux, 6h, 72%; (b) $CH_3I$ (1.5 equiv.), NaH (2.0 equiv.), DMF, reflux, 6h, 78%; (c) HCl (1.0 M) in $Et_2O/CH_2Cl_2$ (1:1), 25°C, 6h, 90%.



**Scheme 3.** Reagents and conditions: (a) $PhCH_2Br$ (1.5 equiv.), $K_2CO_3$ (1.75 equiv.), DMF, 25 °C, 12h, 93%; (b) HAc: $H_2O$ (4:1), reflux, 1.5h, 95%; (c) $PhCH_2Br$ (1.3 equiv.), $K_2CO_3$ (1.5 equiv.), DMF, 25 °C, 12h, 93%; (d) $CH_3I$ (1.2 equiv.), $K_2CO_3$ (1.4 equiv.), DMF, 25 °C, 12h, 94%; (e) Pd/C (10 wt%), $H_2$ (1 atm), THF/EtOH, 8h, 96%.



**Scheme 4.** Reagents and conditions: (a) $Ac_2O$, pyridine, DMAP (0.1equiv.), 25ºC, 12h, 90%; (b) $PhCH_2Br$ (3.0 equiv.), $K_2CO_3$ (7.0 equiv.), KI (1.0 equiv.), acetone, reflux, 6h, 74%; (c) $H_2$ (1 atm), Pd/C (10 wt%), $CH_2Cl_2/Et_2OH$, 12h, 92%; (d) $CH_3I$ (1.5 equiv.), $K_2CO_3$ (1.75 equiv.), DMF, 25ºC, 6h, 90%; (e) NaOH (0.5 M), methanol, $N_2$, 0ºC, 1h, 81%.



**Scheme 5.** Reagents and conditions: (a) $CH_3I$ (3.0 equiv.), $K_2CO_3$ (3.5 equiv.), DMF, 25ºC, 6h, 86%; (b) $H_2$ (1 atm), Pd/C (10 wt%), EtOH, 12h, 91%; (c) $CH_3I$ (1.5 equiv.), $K_2CO_3$ (2.0 equiv.), DMF, 25ºC, 6h, 38% for **7**, 49% for **8**; (d) $CH_3I$ (3.5 equiv.), $K_2CO_3$ (4.0 equiv.), DMF, 25ºC, 8h, 32% for **9**, 54% for **10**.

**Research Highlights**

1. Scutellarin had a wide range of benefits to brain injury.

2. Eight methylated scutellarein derivatives were synthesized based on metabolism in vivo.

3. The thrombin inhibition activity, antioxidant activity and physicochemical properties of these derivatives were evaluated.

4. Preliminary SARs of these derivatives were analyzed.

5. 6-*O*-methyl-scutellarein (**5**) showed good biological activity.