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An efficient, scalable approach to hydrolyze flavonoid glucuronides via activation of glycoside bond

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ABSTRACT

Hydrolyzing flavonoid glucuronides into corresponding aglycones posed some significant challenges. To improve acid-catalyzed hydrolysis process of flavonoid glucuronide, structures of glucuronide, hydrolysis parameters and post-processing were optimized. The optimized condition was performed by hydrolysis flavonoid glycoside methyl ester in a mixed solvent consisting of 2 mol/L H_2SO_4/EtOH/H_2O (1/8/1, v/v/v) at 95 °C for 7 h and resulted in up to 90% aglycone yields, minimal byproduct formations and milder hydrolysis conditions. Furthermore, the optimized method avoids tedious purification steps and is easily conducted on a relatively large-scale using economical and commercially available reagents.

1. Introduction

Flavonoids, a broad class of polyphenolic secondary metabolites from natural source, consist of flavonoid aglycones (FA) and flavonoid glycosides (FG). Variation in the molecular structures of FG originates from the different type of aglycone, sugar and the number of sugar groups, among which the fraction of glucuronide is quite common.[1] Flavonoids have gained much attention from researchers and the food industry due to the fact that they are natural antioxidants as an alternative to synthetic products for the usage in food and cosmetics,[2] as well as their important biological roles in nitrogen fixation and chemical defense.[3] Recent studies also proved that flavonoids possess a broad range of pharmacological properties, including antioxidant, anticancer, and anti-inflammatory activities[4,5]; hence received considerable therapeutic importance.

Except the differences on the structures of flavonoid aglycones and their glycosides, the great bioactive differences are also available. FAs are superior to their corresponding glycosides in various activities, due to the better solubility and oral bioavailability.[6,7] In addition, recent reports indicated that most FGs were hydrolyzed into their corresponding aglycones by human intestinal microflora after oral administration.[8,9] Teruaki et al.[10] found that baicalin could be absorbed as its hydrolyzed products - baicalein but not baicalin itself in the rat gastrointestinal tract. These previous studies show that baicalein possesses more distinctive pharmacological effects and metabolites safety than baicalin, so it is interesting to obtain FA for further chemical modification and preclinical studies. Unfortunately, natural FA is little in contrast to their respective glycosides. Total syntheses of FAs, such as baicalein and scutellarein, have been reported by Silvestri[11] and Chen[12] respectively, but they are unsuitable for industrial production due to their lengthy processes and low yields.

In the light of the natural abundance of FGs,[13] the conversion from FGs to their corresponding aglycones by bio- and chemical-transformations is required urgently. Although bioconversion seems more promising, their application has faced some difficulties, including substrate specificity, enzyme purification,

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0040-4020/

maintenance of enzyme activity and high cost.[14–17] In comparison, the acid catalyzed cleavage of the glycosidic bond has been extensively used for glucosides hydrolysis in view of its universal versatility and maneuverability.[18] Different from glucosides, however, most studies have focused on the acid-catalyzed hydrolyses of glucuronides, because the special structure of glucuronide moiety is so stubborn that glucuronides are hard to be hydrolyzed under mild acid hydrolysis conditions. Much effort has been devoted to develop novel hydrolysis methods of glucuronide, but numerous glucuronides, such as scutellarin and baicalin are still hardly hydrolyzed unless under vigorous acid hydrolysis conditions. Although Li[19] and Tang[20] have independently examined the hydrolysis of scutellarin in aqueous mineral acid recently, both of their methods needed high temperature, long reaction time and without satisfied yields (mostly below 20%). In addition, the formation of byproducts associated with this protocol might occur, which involves an elaborate and tedious procedure; for example, the isomerization of flavonoids is formed due to a reversible Wessely–Moser rearrangement reaction (Fig. 1).[21] Consequently, alternative methods to hydrolyze FGs would be essential for the industrial preparation of FAs, including important chemical constituents in the pharmaceutical, cosmetic, and food industries. Until now, to the best of our knowledge, no appropriate approach on hydrolyzing flavonoid glucuronide into corresponding aglycone with simple purification and high yield processing has been reported.

We hypothesize that the proton of 5-carboxylic acid of flavonoid glucuronide might obstruct the first two steps of acid hydrolysis of glycoside based on theoretical calculations. Therefore, the protection for the carboxyl function in the chemical conversion of FGs to FAs was required. The present study aims to improve dilute-acid hydrolysis of the most FGs by protecting the carboxyl function and to develop a new universally sustainable method to ensure a convenient large-scale production of aglycones. The optimal C_5-substituent groups of glucuronides moiety (hereinafter denoted as FGM) were first chosen based on both the physical properties of glucuronides and the industrial feasibility by theoretical calculation using Gaussian program. The synthetic FGM was then subjected to dilute-acid hydrolysis for FA production. Additionally, the products of the hydrolysis process of FGM were examined and compared with that of glucuronide by the HPLC. The proportional amplification and substrate applicability of this method were also evaluated for the acid hydrolysis of scutellarin and other flavonoid glucuronides.

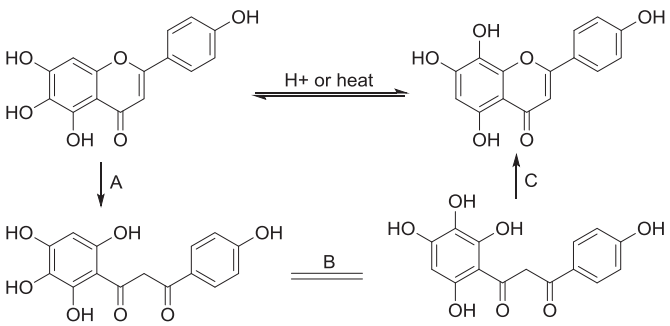


Fig. 1. Mechanism of Wessley-Mosher rearrangement reaction for scutellarein.

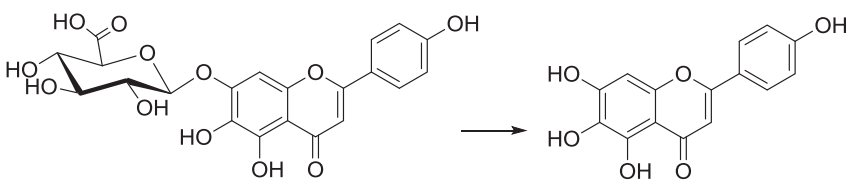
2. Results and discussion

2.1. Cause of glucuronide hydrolysis reaction problems and their solutions

2.1.1. The carboxyl of glucuronide is the "culprit"!

We chose scutellarin as the substrate and attempted to optimize the hydrolysis process in order to improve the demonstrated productivity of scutellarein. It should be noted that 90% EtOH was chosen for economical reason, and H_2SO_4 was used as the acid throughout our study because sulfuric acid can provide strong acidity and stable proton concentration in the acid hydrolysis reaction. Both of concentrations of sulfuric acid and reaction temperatures were evaluated. Indeed, only a 15% conversion was achieved using 0.5 mol/L H_2SO_4 at 120 °C for 48 h (Table 1, entry 5), which was consistent with the previous hydrolyses reaction performed under similar conditions.[20] Using a stronger acidic reaction media could not increase the conversion ratio of scutellarin, while scutellarein isomers might generate due to a reversible Wessely-Moser rearrangement reaction under such hard condition.[24] The rearrangement reaction could happen following several steps: (A) ring opening to the diketone, (B) bond rotation with the formation of a favorable acetylacetone-like phenyl-ketone interaction and (C) ring closure (Fig. 1).

Table 1
Optimization of hydrolysis conditions of scutellarin with sulfuric acid.[a]



Entry	H_2SO_4 (mol/L)	Solvent[b]	T (°C)	Time (h)	Conv. (%)[c]
1	1	90% Ethanol	90	24	4
2	2	90% Ethanol	90	24	5
3	3	90% Ethanol	90	24	8
4	3	90% Ethanol	100	48	10
5	3	90% Ethanol	120	48	15
6	4	90% Ethanol	120	48	16

[a] Unless otherwise stated, all reactions were carried out with scutellarin (0.5 mmol) and H_2SO_4 (15 mmol, **30 equiv**) under N_2 atmosphere.
[b] 90% Ethanol represent EtOH/H_2O = 90/10 (v/v).
[c] Conversion determined by HPLC analysis.

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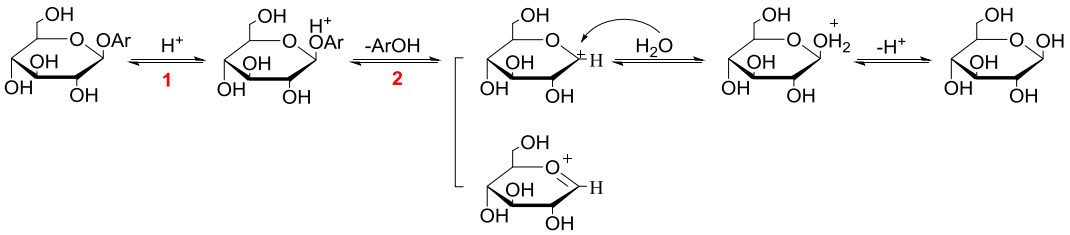

Fig. 2. The universally acknowledged hydrolysis mechanism of glycosides with acid catalyzed, in which the protonation of glycosidic oxygen atom (step **1**) and cleavage of glycosidic bond (step **2**) are the two rate-determining steps in the acid hydrolysis of glucuronide.

In order to find the reason, firstly we have made a detailed analysis of the acid hydrolysis mechanism of glycoside. As the mechanism of acid hydrolysis reaction of glycoside showed in Fig. 2, the first step is protonation at the exocyclic oxygen of the glycosidic bond affording the conjugate acid and attaining equilibrium rapidly, followed by loss of the aglycon with the formation of a cyclic carbonium.[22] The second step is rate-determining and shows characteristics of an S_N1-type substitution reaction which depends on the stability of the oxo-carbenium ion formed upon the departure of the protonate leaving group as an alcohol.[23] The conjugate acid can advance to this rate-determining step. On the basis of these characteristics, protonation of the glycosidic oxygen atom (hereinafter denoted as O_e), *viz.* concentration of the conjugate acid, and rate-limiting cleavage of the protonated glycosidic bond are the practical rate-determining parameters during the acid hydrolysis process.[18] It has been accepted that this accessibility of glycosidic bonds is hindered by the physical structure of glucuronide because of a free carboxyl function in their glucuronide moiety.[22] As a result, protonation of the glycosidic oxygen atom and cleavage of the glycosidic bond are the rate-determining steps in the acid hydrolysis of glycoside. The effect of carboxyl group on the key steps for acid hydrolysis of glucuronide will be figured out.

To understand the process of acid hydrolysis of glycosides, we studied the protonation abilities of various types of sugar ligands. As shown in Fig. 3A–C, 2-amino glucoside (A) have been reported to be acid-hydrolyzed more rapidly than 2-deoxy-glucoside (B) under the same reaction conditions.[22] A possible explanation for the slower hydrolysis rate of 2-amino glucoside (A) is that the amino of sugar unit can not only compete with the exocyclic oxygen of the glycosidic bond when it combined with a proton but also be protonated to generate electron-withdrawing inductive effect, which leads to a reduction of the electron cloud density at the terminal carbon atom of sugar unit (hereinafter be denoted C_t) as well as weakens the protonation ability of the O_e at the glycosidic bond. In contrast, for 2-deoxy-glucoside (B), without the effect of the amino at position 2 of glucoside, the electron cloud density of the C_t became even higher, which could also explain that 2, 6-dideoxy glucoside could be hydrolyzed just using 0.02–0.05 mol/L HCl in EtOH-H_2O system.[25] Certainly, the aromatic aglycone has π-electron conjugation system, which could also reduce the electron density of Oe. But, the effect of the conjugation on the Ct is relatively weak. Most of aromatic-aglycone-containing glucosides, such as daidzin, can be hydrolyzed to aglycone daidzein under conventional acid hydrolysis conditions.[26] Above all, we believe that the difficulty of hydrolyzing the aromatic-aglycone-containing glucuronide (C) might be attributed mainly to its 5'-carboxy group for its steric hindrance and H-bonded to glycosidic oxygen, impeded the proton attack of the O_e. Besides, 5'-carboxy group's strong electron-withdrawing inductive effect could reduce the stability of the oxo-carbenium ion formed upon the glycosidic bond cleavage since protonation will facilitate the electron flow as symbolized by the arrows in these formulas, which makes aromatic-aglycone-containing glucuronides difficult to be hydrolyzed by dilute acid. Therefore, our study suggests that the proton of 5'-carboxylic acid might be the "culprit"!

2.1.2. *Optimization of C_5-substituent groups of aromatic-aglycone-containing glucuronide*

Next, we need to seek a solution in order to reduce the adverse effects for the glucuronide hydrolysis caused by the carboxyl group. Firstly, we tried to hydrogenate the 5-carboxyl of scutellarin into a hydroxyl in order to eliminate the effect of the carboxyl proton. But, this method was finally abandoned, considering the strict reaction conditions required and high cost of some reducers, such as lithium aluminum hydride or borane. Inspired by the application of protective groups in organic chemistry,[27] we planned to accelerate the hydrolysis reaction by protecting the 5-carboxyl with electron-donating groups, which could inhibit the protonation of 5-carboxyl and thereby might reduce its electron-withdrawing effect on the C_t (Fig. 3D).

The choice of the protecting groups mainly depended on their molecular size, electronic effects and operability.[28] On the base of the advantage of alkanes on chemical stability, electron-donating ability and modified difficulty, finally, ethyl or methyl was selected as the protective group. The aim of chemical modification with alkanes, which has methyl donor groups, is to weaken the adverse effects from the 5-carboxyl and to obtain excellent yield of aglycones with a mild acid hydrolysis conditions. We found that methoxycarbonyl, an electron-donating group, is inductively less electronegative than carboxy group and we proposed that the introduction of the methoxycarbonyl group onto the 5-carboxyl, through accumulating electron density at the terminal carbon, can enhance both protonation of pre-equilibrium and rate-limiting formation of oxo-carbenium ion intermediate. Therefore, we

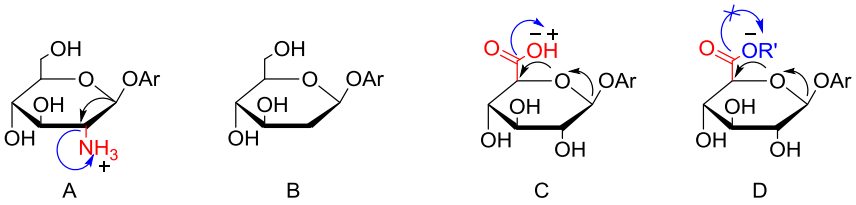

Fig. 3. The protonated capability of different glycosides bond oxygen atoms of aromatic-aglycone-containing glucuronides.

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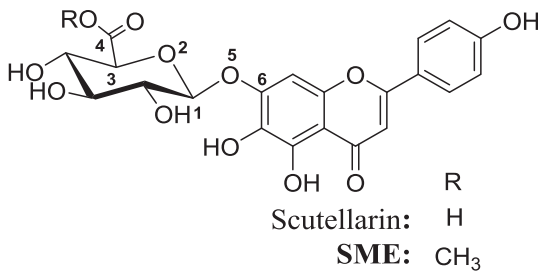
hypothesize that the dual effects effectively accelerate the hydrolysis rate of glucuronide.

To prove this hypothesis, the theoretical calculation studies were used for the acid hydrolysis pathway and mechanism of scutellarin, because it can provide valuable information to figure out the key factors which affect the hydrolysis rate of scutellarin. Density functional theory (DFT) has nowadays become general tool to the understanding and predicting a broad range of chemical, physical, and biological phenomena featuring the realm of coordination chemistry. It was noted that functional Becke's three-parameter hybrid exchange functional and the Lee–Yang–Parr non-local correlation functional (B3LYP) is the most widely used and has been proven to yield satisfactory results on energy and geometry, but with a low computational cost.[29] More recent studies on mechanism show that DFT method employing B3LYP successfully explains mechanistic features of the organic system as well.[30] Therefore, to confirm this hypothesis and further understand the acid hydrolysis reaction, Gaussian09 program with B3LYP and the 6-31 + G* basis set was employed for the computational studies on the acid hydrolysis mechanism of glucuronide.[31] Scutellarin, a glucuronide with the highest level from the traditional Chinese medical herb *Erigeron breviscapus* (vant.) Hand.-Mazz, and its modified scutellarin: scutellarin methyl ester (**SME**) were representatively selected for the subsequent experiments. The optimized structures of scutellarin and **SME** were displayed in Fig. S3 (see ESI for details).

Table 2
The average atomic charges of scutellarin and the modified **SME** at the B3LYP/6-31 + G(d) Level and their ^{13}C NMR Data (75 MHz in DMSO-d_6).



Atoms	Scutellarin		**SME**	
	Charges	δ_C	Charges	δ_C
C1 (**C_t**)	0.35635	93.55	0.13757	92.53
O2	−0.41228	–	−0.31482	–
C3	0.02401	75.45	−0.13204	73.19
C4	0.69501	170.04	0.59146	169.70
O5	−0.326363	–	−0.15769	–
C6	0.36953	161.13	0.30161	151.39

2.1.2.1. The influence of methyl group on the protonation at O_e of aromatic-aglycone-containing glucuronides. Atomic charges were obtained by natural population analysis with Gaussian09.[32] Table 2 presented the average atomic charge distribution of **SME** and less positive charges of C_t in **SME** (0.13757 |e|) than that of scutellarin (0.35635|e|), while in the ^{13}C NMR spectrum (Table 2), the C_t signal resonances of **SME** (δ_C 92.53) is in higher field region than that of scutellarin (δ_C 93.55), which preliminarily suggested the higher electron density of the C_t of **SME** than that of scutellarin, which indicated that the glycosidic oxygen atom of **SME** protonated with dilute acids more easily. Furthermore, mentioned the hydrolysis of scutellarin, the calculation result revealed that the hydrogen-bonding arrangement between the oxygen of glycoside bond and the carboxyl hydrogen of glucuronide moiety at a distance of 2.05 Å, which hampered the interaction between proton and O_e (Fig. 4, INT-1). In contrast to scutellarin, the methoxycarbonyl group of **SME** was too far away from the glycoside bond to interfere with the protonation of O_e during the hydrolysis process (Fig. 4, INT-2). Therefore, the increase of electron density on the C_t and easier protonation of O_e, caused by the methyl esterification, accelerated the first step of hydrolysis, which partially insinuated that **SME** was much easier hydrolyzed than scutellarin under dilute acid conditions.

2.1.2.2. Effects of methyl group on the cleavage of the glycoside bond of aromatic-aglycone-containing glucuronides. Analysis of the hydrolysis paths given in Fig. 2 indicates that this reaction takes place through a stepwise mechanism. To shed further light on the acid hydrolysis mechanism, the transition states (TSs) with reasonable energy were identified. Thus, we attempted to optimize the conformation of compounds and compare the activation energy barrier ΔE from reactants (INT-1 and INT-2) with their corresponding transition states (TS-1 and TS-2) to determine whether the methyl group can also accelerate the second step of acid hydrolysis reaction. DFT calculations indicate that the TS-1 has an activation energy barrier of 206.2 kcal/mol relative to the starting material in ethanol, and the TS-2 has the lowest energy barrier of only 128.6 kcal/mol, which is 77.6 kcal/mol lower in energy than

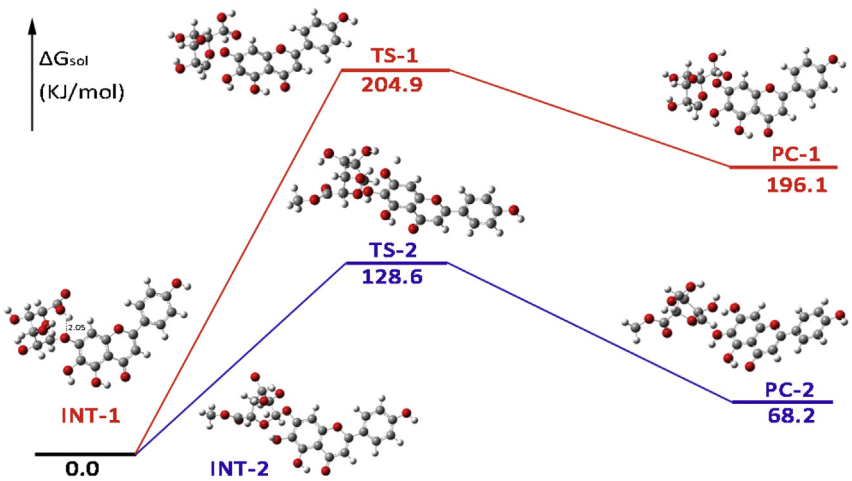


Fig. 4. Energy profile for a simplified model of acid hydrolysis for scutellarin (red) and **SME** (blue) using B3LYP/6-31 + G* methods.

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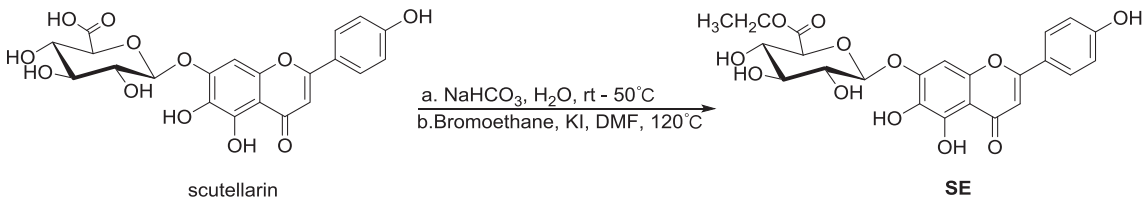
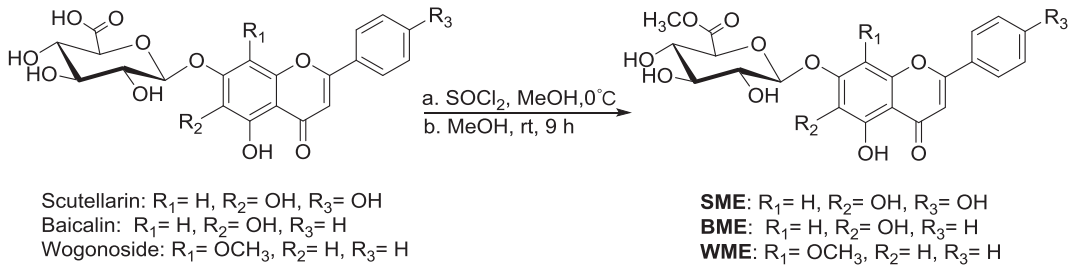
that of TS-1 (Fig. 4). On the other hand, the activation enthalpy associated with cleavage of the glycosidic bond of conjugate acid INT-1 to form the corresponding cyclic cation intermediate PC-1 is 203.7 kcal/mol. The more stable the oxo-carbenium ion formed is, the more easily the corresponding glycoside bond can be broken.[33] Consistented with the theoretical prediction from the chemical point of view, the conversion of INT-2 to PC-2 has an enthalpic barrier height of only 76.4 kcal/mol (Table S1 in the ESI), which suggested that the oxacarbenium-ion intermediate PC-2 is more stable than PC-1, as a consequence of the electron-releasing character of the methyl group present in the glucuronide moiety of intermediate INT-2.

Therefore, the designed alkyl ester groups might accelerate the second step of acid hydrolysis reaction by reducing the activation energy barrier ΔE and enthalpic barrier height ΔH in the hydrolysis reaction of **SME**, which further indicated that the acid hydrolysis of **SME** was faster than that of scutellarin and also verified the designed rationality of **SME**. Finally, ethyl or methyl was selected as the protective group.

Ethyl and methyl groups were connected to 5-carboxyl via ester bond. The scutellarin ethyl (**SE**) was prepared by reacting scutellarein with $NaHCO_3$ to generate scutellarin sodium salt, followed by adding stoichiometric amount of bromoethane in the presence of potassium iodide in N, N-dimethylformamide (DMF), a high boiling toxic solvent, at 120 °C under nitrogen atmosphere (Scheme 1).[34] Yellow powder of SE (21%) was obtained through tedious post-processing steps. In contrast, the preparation of **SME** was easier and faster in multiple aspects. We found that thionyl chloride dropped in methanol under ice bath quickly generated chlorosulfurous acid methyl ester (**CAME**), a methyl ester reagent, which could methyl esterize carboxyl chemo-selectively but not methylate the phenolic hydroxyl at room temperature. Considering this favorable property, **SME** was obtained from scutellarin treated with excess **CAME** (Scheme 2) in a quantitative yield without further purification (Fig. 5A). The productivity and purity of **SME** were determined by HPLC, ^{1}H NMR (Fig. S2 in the ESI), ^{13}C NMR (Fig. S3 in the ESI) and



Scheme 1. Synthesis of ethyl ester of scutellarin.



Scheme 2. Synthesis of methyl ester of glucuronide.

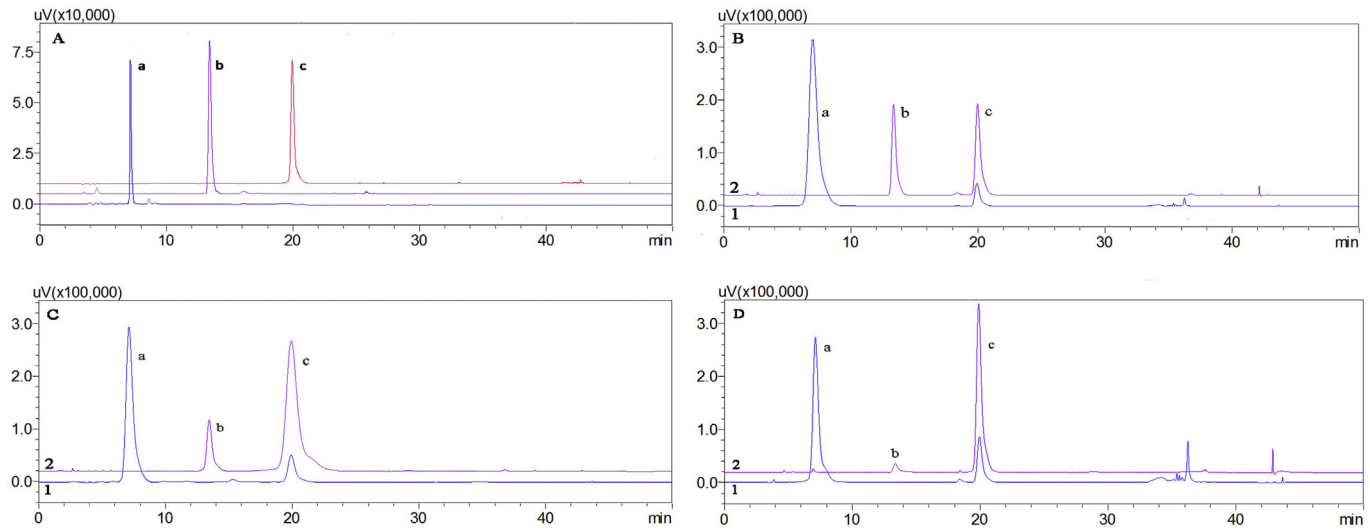


Fig. 5. HPLC chromatograms of scutellarin.[a] (A) Standards: a. Scutellarin; b. **SME**; c. Scutellarein; (B) scutellarin (1) and **SME** (2) hydrolyzed for 2 h; (C) scutellarin (1) and **SME** (2) hydrolyzed for 4 h; (D) scutellarin (1) and **SME** (2) hydrolyzed for 6 h.

[a] All reactions were carried out with scutellarin (0.5 mmol) or SME (0.5 mmol) and 2 mol/L H_2SO_4 (2.5 mmol) in 90% EtOH at 95 °C under N_2 atmosphere.

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ESI-MS (Fig. S4 in the ESI). Compared with **SE**, the synthesis of **SME** exhibited advantages in four areas: solvent, temperature, post-treatment and yield. Further, the acid hydrolysis of **SME** has a higher conversion than that of **SE** under the same reaction conditions (the data not shown). As a consequence, methyl was confirmed to be the most promising protective group, and the hydrolysis process of **SME** could be conscientiously evaluated.

2.2. Successful hydrolysis of scutellarin and its excellent applicability

2.2.1. Optimization of the hydrolysis parameters of SME

Initially, the acid hydrolysis of **SME** was performed under mild conditions. It was cheerful to find that the hydrolysis reaction of **SME** could proceed in the presence of 0.5 mol/L H_2SO_4 at 90 °C for 12 h, yielded 61% of scutellarein (Table 3, entry 1), which indicated that it was crucial to protect the carboxyl for an efficient acid hydrolysis of scutellarin. Encouraged by this result, we optimized the top two most important hydrolysis parameters[35] - the concentration of sulfuric acid (0.5–3 mol/L) and the reaction temperature (90–100 °C) (Table 3). To our delight, among the different conditions explored in the optimization, scutellarein was obtained in a 95% yield when the concentration of H_2SO_4 was elevated to 2 mol/L at 100 °C (Table 3, entry 6). However, the conversion was not further increased at higher concentration of H_2SO_4 (95%, entry 7), so rather harsh acidic conditions have not been evaluated. Interestingly, lowering the temperature to 95 °C resulted in a slightly higher yield (96%, entry 8), which may be due to the instability of the ester bond at high temperature. Finally, the optimum hydrolytic reaction of **SME** was performed in 2 mol/L H_2SO_4/EtOH: H_2O (9:1) for 7 h.

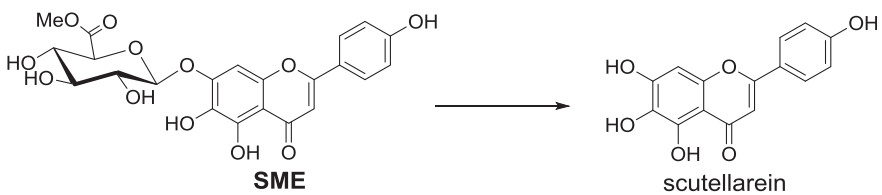
With the optimum conditions, we further investigated the hydrolysis procedure of **SME** compared with scutellarin. The transformation of scutellarin and **SME** could be readily monitored, comparing the peak area of the hydrolysates with that of the standards (Fig. 5A). The results are shown in Fig. 5B D. For the conversion of scutellarin, only 6.9% yield of the desired product was detected at 6 h (Fig. 5B and D). Excitingly, the same condition could successfully convert **SME** into scutellarein within 6 h and no isomer was detected (Fig. 5D). The cleavage of ester bond of **SME** under acidic hydrolysis conditions did not happen, which was confirmed by HPLC. Eventually, the yield of scutellarein could reach 96% with only traces of **SME** remaining by HPLC (Fig. 5A and b) and ^{1}H NMR (Fig. S5 in the ESI). Therefore, no further purification was required.

2.2.2. The proportional amplification and substrate applicability of this method

Notably, both methyl esterification of scutellarin and its hydrolysis are scaled up which proved to be a practical route for the preparation of scutellarin. In our study, scutellarin in 10-g scale (10.01 g) was subjected to react with **CAME** to obtain **SME**, which was further hydrolyzed under the optimized conditions for 9 h (Scheme 3), then fine yellow powder of scutellarein (5.57 g, 90% yield) was obtained after a facile purification procedure.

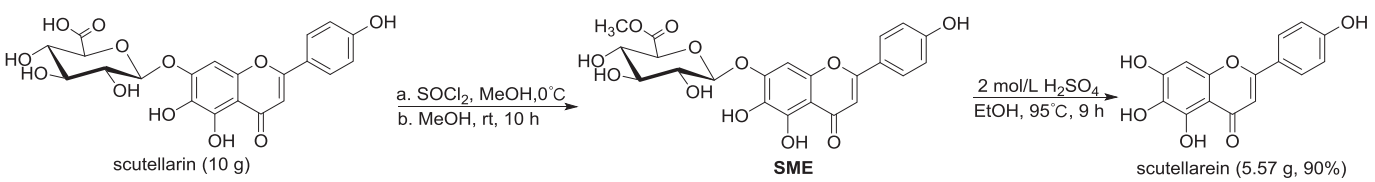
To efficiently obtain other pharmaceutically valuable aglycones, we applied these selected conditions to the corresponding flavonoid glucuronides which are widely distributed in plants and readily available, such as baicalin and wogonoside.[36] Gratifyingly, baicalein was obtained by hydrolyzing baicalin methyl ester (**BME**) in an excellent yield of 97%, and the selected conditions were also

Table 3
Optimization of hydrolysis conditions of **SME** with sulfuric acid.[a]



Entry	H_2SO_4 (mol/L, equiv.)	T (°C)	Time (h)	Conv. (%)[b]	Yield (%)[c]
1	0.5, 5	90	12	69	61
2	0.5, 10	90	12	71	66
3	1, 10	90	10	82	75
4	1, 10	100	10	86	80
5	1, 10	100	15	88	83
6	2, 10	100	10	98	95
7	3, 10	100	10	99	95
8	2, 10	95	7	98	96
9	2, 5	95	7	98	96
10	2, 5	90	7	94	91

[a] Unless otherwise stated, all reactions were carried out with scutellarin (0.5 mmol) in 90% ethanol under N_2 atmosphere.
[b] Conversion determined by HPLC analysis.
[c] Isolated yield.



Scheme 3. 10-Gram scale acid hydrolysis of scutellarin.

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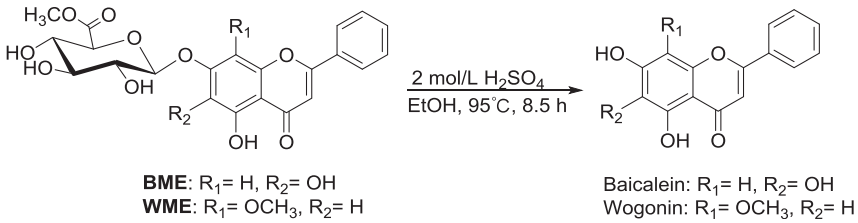


Scheme 4. Acid hydrolysis of **BME** and **WME**.

applied for the preparation of wogonin from wogonoside without any difficulties (Scheme 4).

For the aliphatic-aglycone-containing glucuronides, such as glycyrrhizic acid, the glycosidic bond without π-electron conjugation system is less stable and easily hydrolyzed at room temperature. But, we speculate that the cleavage of the glycosidic bond to produce glycyrrhetinic acid is more likely occurred by alcoholysis rather than hydrolysis reaction in the presence of HCl/MeOH at room temperature.[37] In order to verify this conjecture, we operated four sets of comparative experiments (Table 4).

As shown in Table 4, the hydrolytic yield of glycyrrhizic acid was relatively low, only a 14% conversion was achieved under 6 M HCl at 20 °C (entry 1). The methanolysis reaction of glycyrrhizic acid went very smoothly in the presence of hydrogen chloride which was furnished via methanol and thionyl chloride/acetyl chloride (90% and 92%, entry 2 and 3). Glycyrrhizic acid can also be subjected to ethanolysis at a lower yield compared with the methanolysis because of the weaker reactivity of ethanol than methanol (71%, entry 4).

3. Conclusions

We have developed an efficient approach to obtain scutellarein by hydrolyzing the methyl-esterification product of scutellarin, which has been successfully applied in our laboratory. This protocol has possessed the advantages of mild condition, simple operation and excellent yield. It is worth noting that the present methodology can be readily scaled up and represents a practical route to prepare scutellarein, as well as successfully prepare other biologically important bioactive flavone aglycones.

4. Experimental section

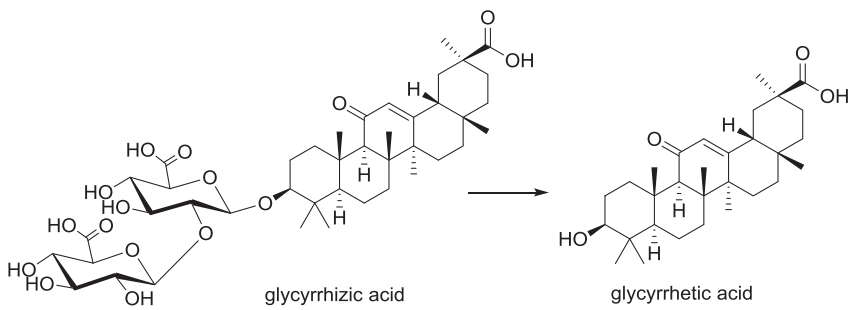
4.1. Materials

Baicalin (purity > 99%), scutellarin (purity > 99%), wogonoside (purity > 98%) and glycyrrhizinate (purity > 99%) were purchased from Aladdin Reagent Company. Acetonitrile (HPLC grade) was purchased from Tedia Company, Inc. (USA). Unless otherwise specified, all the solvents and reagents were analytical grade and obtained from commercial suppliers. Solvents were purified as per the procedures given in the "*Text book of practical organic chemistry*" by Vogel (6th Edition). Water was purified with a Millipore Milli Q-Plus system (Millipore, MA, USA). All the reactions were performed under a nitrogen atmosphere unless otherwise noted.

4.2. Computational details

The structures corresponding to the reactants, transition states [TSs], intermediates, and the products for the hydrolysis reaction were optimized, using the GAUSSIAN 09 computational package with DFT methods as implemented in the computational package.[31] All calculations were carried out at the B3LYP level using the 6-31G (d,p) basis set. The compounds were also optimized using the same

Table 4
The alcoholysis reaction of glycyrrhizic acid.[a]



Entry	Acid	Solvent	T (°C)	Time (h)	Yield (%)[f]
1	6 M HCl[b]	Ethanol	20	8	14
2	HCl[c]	Methanol	20	8	90
3	HCl[d]	Methanol	20	8	92
4	HCl[e]	Ethanol	20	8	71

[a] Reaction conditions: all of the reactions were performed with glycyrrhizic acid (82.2 mg, 0.1 mmol) and acid in solvent (2 mL).
[b] 5mol/L HCl (3 mmol, 30 equiv).
[c] Thionyl chloride (1.2 mmol, 90 μL) was slowly added to anhydrous methanol (2 ml) at 0 °C for 1 h.
[d] Acetyl chloride (1.2 mmol, 86 μL) was slowly added to anhydrous methanol (2 ml) at 0 °C for 1 h.
[e] Acetyl chloride (1.2 mmol, 86 μL) was slowly added to anhydrous ethanol (2 ml) at 0 °C for 1 h.
[f] Yield of isolated product.

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functional and basis set in EtOH as the solvent based on the Polarizable Continuum Model (PCM) which is developed by Tomasi et al.[38] The vibration frequencies were calculated at the same level to confirm that whether the structures correspond to energy minima or transition states (zero or one imaginary frequencies, respectively).

4.3. Analytical methods

^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance 300 MHz spectrometer at 300 K, using dimethyl sulfoxide (DMSO-d_6) as solvent and tetramethylsilane (TMS) as an internal standard. Mass spectra were measured on Waters Q-TOF Micro™ using electrospray ionization (ESI). Melting points were determined on a RY-1 micro melting point apparatus (Tianjin Analytical Instrument Corp., Tianjin, China). Analytical thin-layer chromatography (TLC) were performed on silica gel GF/UV 254 and visualized under UV light at 254 and 365 nm.

Analysis of hydrolysis was performed with a Shimadzu LC-2010/CHT system equipped with quaternary gradient pump, auto sampler, column oven and UV detector. The data were processed by LC-solution software which was used for instrument control and data acquisition. The separation was carried out on a phenomenex Gemini C18 column (4.6 mm × 250 mm, 5 μm). The column temperature was set at 25 °C. The mobile phase consisted of acetonitrile and 1% formic acid water in the ratio of 76:24 (v/v) with isocratic elution. The flow rate was 1.0 mL/min. Analytes were monitored at 254 nm. The injection volume was 10 μL.

4.4. Preparation of scutellarin ethyl ester (SE)

Scutellarin (2 mmol, 924 mg) was dissolved in 10 mL water, then sodium bicarbonate (2 mmol, 168 mg) was added. The solution was stirred 0.5 h at room temperature, then stirred at 50 °C for 0.5 h. After the completion of the reaction, water was evaporated under reduced pressure. Scutellarin salt was obtained. A mixture of scutellarin salt (1 mmol, 484 mg), bromoethane (3 mmol, 224 μL) and KI (0.1 mmol, 16.6 mg) in DMF (3 mL) was stirred at 120 °C for 3 h under a N_2 atmosphere. Then the reaction mixture was poured into water (30 mL) and extracted with ethyl acetate (3 × 15 mL). The organic layer was separated, washed with water (2 × 10 mL) and dried over anhydrous sodium sulfate. Organic solvent was concentrated under vacuum, recrystallization with ethyl acetate to yield **SE** as a yellow powder. Yield 21%; mp 259–261 °C; ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm): 12.79 (1H, s, 5-OH), 10.37 (1H, s, 6-OH), 8.63 (1H, s, 4′−OH), 7.91 (m, 2H, 2′−H, 6′-H), 7.10 (1H, s, 8-H), 6.92 (2H, d, J = 7.4 Hz, 3′−H, 5′-H), 6.81 (1H, s, 3-H), 5.46 (2H, m, 1″−H, 5″-H), 5.28 (2H, m, −CH_2−),4.13 (3H, m, 2″−H, 3″−H, 4″-H), 2.50–3.46 (3H, m, other sugar protons), 1.22 (3H, t, J = 4.4 Hz, −CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz, δ ppm): 183.0, 168.9, 163.8, 160.5, 152.1, 150.3, 148.7, 129.9, 129.2, 121.4, 115.9, 106.0, 103.4, 100.7, 93.1, 74.9, 74.5, 73.2, 71.3, 60.5, 15.3; MS (ESI) m/z = 489.1 [M−H]$^-$. The melting points and ^{1}H NMR were in good agreement with the reported.[33]

4.5. General procedure: preparation of glucuronide methyl ester

To a stirred solution of methanol (19 ml) in an ice both was added thionyl chloride (900 μL) drop wise over a period of 1 min. After complete addition, the solution was stirred 1 h at room temperature to give chlorosulfurous acid methyl ester. Then glucuronide (0.1 eq) was added. The mixture was stirred at room temperature for the indicated time. After the completion of the reaction, solvents were removed under reduced pressure and the desired products were obtained.

4.5.1. Scutellarin methyl ester (SME)

Following the general procedure with scutellarin (1 mmol, 462 mg), the mixture was stirred at room temperature for 9 h. Methanol was removed and **SME** (455 mg, 95.6%) was obtained without further purification as a yellow powder; mp 251–252 °C; ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm) δ 12.85 (s, 1H, 5-OH), 10.39 (s, 1H, 4′−OH), 7.93 (d, J = 9.0 Hz, 2H, H-2′, H-6′), 7.00 (s, 1H, H-8), 6.94 (d, J = 9.0 Hz, 2H, H-4′, H-5′), 6.81 (s, 1H, H-3), 5.28 (d, J = 7.0 Hz, 1H, H-1″), 4.20 (d, J = 6.0 Hz, 1H, H-2″), 3.70–3.90 (3H, m, other sugar protons), 3.68 (3H, s, −OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz, δ ppm): 182.8, 169.7, 164.5, 161.6, 151.3, 149.4, 147.3, 130.8, 128.9, 121.7, 116.4, 106.3, 102.9, 100.2, 93.9, 75.7, 75.4, 73.1, 71.8, 52.4 (−OCH_3); MS (ESI) m/z = 477.1 [M+H]$^+$, 475.1 [M - H]$^-$; HRMS (ESI): m/z calcd for $C_{22}H_{19}O_{12}$: 475.0882; found: 475.0868 [M−H]$^-$.

4.5.2. Baicalin methyl ester (BME)

Following the general procedure with baicalin (1 mmol, 446 mg), the mixture was stirred at room temperature for 8.5 h. Methanol was removed and **BME** (442 mg, 96.1%) was obtained without further purification as a yellow powder; mp 197–199 °C; ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm) δ 12.60 (s, 1H, 5-OH), 8.69 (s, 1H, 6-OH), 8.07 (m, 2H, H-2′, H-6′), 7.62 (m, 3H, H-3′, H-4′, H-5′), 7.06 (s, 1H, H-3), 7.01 (s, 1H, H-8), 5.52 (m, 2H, sugar hydroxy), 5.30 (d, J = 6.3 Hz, 1H, H-1″), 4.22 (d, J = 6.0 Hz, 1H, H-2″), 3.68 (3H, s, −OCH_3), 3.40–3.43 (3H, m, other sugar protons); ^{13}C NMR (DMSO-d_6, 75 MHz, δ ppm): 182.5, 169.1, 163.5, 151.2, 149.1, 146.7, 132.0, 130.8, 130.5, 129.1, 126.3, 106.1, 104.7, 99.7, 93.6, 75.5, 75.0, 72.7, 71.3, 51.9 (−OCH_3); MS (ESI) m/z = 459.1 [M - H]$^-$; HRMS (ESI): m/z calcd for $C_{22}H_{19}O_{11}$: 459.0933; found: 459.0917 [M−H]$^-$.

4.5.3. Wogonoside methyl ester (WME)

Following the general procedure with wogonoside (1 mmol, 460 mg), the mixture was stirred at room temperature for 8.5 h. Methanol was removed and **WME** (443 mg, 96.4%) was obtained without further purification as a yellow powder; mp 241–243 °C; ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm) δ 12.56 (s, 1H, 5-OH), 8.04 (m, 2H, H-2′, H-6′), 7.59 (m, 3H, H-3′, H-4′, H-5′), 7.02 (s, 1H, H-6), 6.72 (s, 1H, H-3), 5.35 (d, J = 6.1 Hz, 1H, H-1″), 4.23 (d, J = 7.3 Hz, 1H, H-2″), 3.40–3.46 (3H, m, other sugar protons), 3.89 (s, 3H, 8-OCH_3), 3.68 (s, 3H, 5″−OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz, δ ppm): 182.7, 169.7, 163.9, 157.8, 156.4, 150.1, 132.6, 131.2, 129.7, 128.1, 126.8, 105.8, 105.4, 99.9, 98.9, 76.0, 75.5, 73.3, 71.7, 61.8, 52.5 (−OCH_3); MS (ESI) m/z = 473.1 [M−H]$^-$; HRMS (ESI): m/z calcd for $C_{23}H_{21}O_{11}$: 473.1089; found: 473.1102 [M−H]$^-$.

4.6. Acid hydrolysis of scutellarin

In a 10 mL round-bottomed flask, 300 mg (0.65 mmol) of scutellarin was dissolved in 5 mL of specific ethanol. To the mixture was added 1 mL of sulfuric acid with a specific concentration (Table 1), and the solution was stirred and heated to a specific temperature (Table 1). The reaction was timed just after the addition of H_2SO_4. After completion of the reaction, aliquots of the reaction mixture were subjected to HPLC analysis. The mobile phase for HPLC consisted of solvent (A) i.e., and (B), solvent i.e. acetonitrile. The mobile phase consisted of acetonitrile and 0.1% (v/v) formic acid in filtered MilliQ water in the ratio of 24: 76 (v/v) with isocratic elution. The flow rate was 1.0 mL/min. Analytes were monitored at 254 nm. The injection volume was 10 μL.

4.7. General procedure: acid hydrolysis of glucuronide methyl ester to obtain corresponding aglycones

To a stirring mixture of glucuronide methyl ester (0.5 mmol) and sulfuric acid (1.1 mL) in ethanol (8 mL) was added water (1.1 mL),

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the reaction mixture was stirred at 95 °C for the indicated time under a N_2 atmosphere. After cooled down to the room temperature, the mixture was poured into water. Then the precipitate was filtered off, washed with water and recrystallized from methanol to obtain the desired products.

4.7.1. Scutellarein

137 mg, 96% yield; yellow powder; mp: 325–328 °C; ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ 12.79 (s, 1H, 5-OH), 10.47 (s, 1H, 7-OH), 10.32 (s, 1H, 4′–OH), 8.75 (s, 1H, 6-OH), 7.89 (d, J = 8.5 Hz, 2H, H-2′, H-6′), 6.90 (d, J = 8.5 Hz, 2H, H-3′, H-5′), 6.73 (s, 1H, H-8), 6.56 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 75 MHz, δ ppm): 182.2, 163.7, 161.2, 153.4, 149.9, 147.2, 129.3, 128.5, 121.6, 116.2, 104.2, 102.4, 94.1; MS (ESI) m/z = 285.1 [M−H]$^-$.

4.7.2. Baicalein

131 mg, 97% yield; yellow powder; mp: 263–265 °C; ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ 12.65 (s, 1H, 5-OH), 10.57 (s, 1H, 7-OH), 8.82 (s, 1H, 6-OH), 8.05 (d, J = 8.2 Hz, 2H, H-2′, H-6′), 7.55–7.58 (m, 3H, H-3′, H-4′, H-5′), 6.93 (s, 1H, H-8), 6.62 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 75 MHz, δ ppm): 182.0, 162.7, 153.5, 149.7, 146.9, 136.6, 130.8, 129.2, 128.9, 126.1, 104.3, 104.2, 93.9; MS (ESI) m/z = 269.1 [M−H]$^-$.

4.7.3. Wogonin

136 mg, 96% yield; yellow powder; mp: 204–205 °C; ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ 12.48 (s, 1H, 5-OH), 10.82 (s, 1H, 7-OH), 8.02–8.04 (m, 2H, H-2′, H-6′), 7.57–7.58 (m, 3H, H-3′, H-4′, H-5′), 6.94 (s, 1H, H-6), 6.29 (s, 1H, H-3), 3.84 (s, 3H, 8-OCH$_3$); ^{13}C NMR (DMSO-d_6, 75 MHz, δ ppm): 181.9, 162.8, 157.3, 156.2, 149.5, 131.9, 130.7, 129.1, 127.6, 126.1, 104.9, 103.7, 99.1, 60.9; MS (ESI) m/z = 283.1 [M−H]$^-$.

4.8. Alcoholysis of glycyrrhizinate

Thionyl chloride (12 mmol, 900 μL) was slowly added to a solution of anhydrous methanol (20 ml) at 0 °C. After 1 h stirring, glycyrrhizinate (1 mmol, 823 mg) was added. The reaction mixture was allowed to warm up to room temperature and stirred for 8 h. Methanol was then removed under reduced pressure and the resulting solid was filtered off, washed with water and pentane then dried to afford glycyrrhetic acid as a white powder. (425 mg, 90%). mp 263–265 °C; ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ 5.31 (d, 1H, J = 7.2 Hz), 3.00 (m, 1H), 1.87 (m, 1H), 1.34, 1.26, 1.25, 1.22, 1.14, 1.08 and 0.95 (s, seven methyl groups); ^{13}C NMR (DMSO-d_6, 75 MHz, δ ppm): 199.1, 177.7, 170.0, 127.3, 71.58, 61.1, 54.3, 48.1, 44.9, 43.1, 42.9, 37.6, 36.4, 32.1, 31.6, 30.4, 28.4, 28.2, 28.1, 27.8, 27.1, 26.1, 25.8, 23.1, 23.0, 18.4, 17.0, 16.2, 16.1, 15.9; MS (ESI) m/z = 269.1 [M−H]$^-$.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2017.02.039.

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