European Journal of Medicinal Chemistry 54 (2012) 210–222

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Metabolism-based synthesis, biologic evaluation and SARs analysis of *O*-methylated analogs of quercetin as thrombin inhibitors

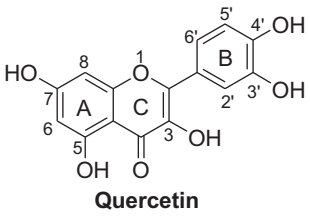
Zhi-Hao Shi [a,c,1], Nian-Guang Li [a,b,*,1], Yu-Ping Tang [a,**], Wei-Li [a,b], Lian-Yin [a], Jian-Ping Yang [a], Hao-Tang [a], Jin-Ao Duan [a,**]

[a] Jiangsu Key Laboratory for High Technology Research of TCM Formulae, Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210046, China
[b] Department of Medicinal Chemistry, Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210046, China
[c] Department of Organic Chemistry, China Pharmaceutical University, Nanjing, Jiangsu 211198, China

HIGHLIGHTS

- Flavonoids maybe promising lead compounds as thrombin inhibitors.
- 17 methylquercetin derivatives were synthesized based on metabolism *in vivo*.
- The thrombin inhibition activity of 17 methylquercetin derivatives were evaluated.
- Preliminary SARs of these derivatives as thrombin inhibitors were analyzed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 April 2012
Received in revised form
26 April 2012
Accepted 30 April 2012
Available online 14 May 2012

Keywords:
Quercetin
Metabolism
Thrombin
Structure-activity relationship

ABSTRACT

In blood, quercetin is mainly found in metabolized forms. In order to study the activities of these quercetin metabolites in cardiovascular disease, 17 methylquercetin derivatives were synthesized based on metabolism *in vivo*, their thrombin inhibition activity were evaluated through the analyzation of prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB). The results showed that 6 methylquercetin derivatives had stronger inhibitory activities than that of quercetin. Preliminary SARs analysis showed that hydroxyl groups at C-3′ and C-4′ position in the B-ring and hydroxyl group at C-3 position in the C-ring played key roles in the thrombin inhibitory activity. The findings of this study would provide information for the exploitation and utilization of quercetin as thrombin inhibitor for thrombotic disease treatment.

1. Introduction

Nowadays, thrombotic diseases are major causes of mortality and morbidity. In thrombotic diseases, thrombin is generated in response to vascular injury, it acts as a multifunctional serine protease and catalyzes the proteolytic cleavage of the soluble plasma-protein fibrinogen to form insoluble fibrin leading 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.

* Corresponding author. Jiangsu Key Laboratory for High Technology Research of TCM Formulae, Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210046, China. Tel./fax: +86 25 85811916.
** Corresponding authors. Tel./fax: +86 25 85811916.
E-mail addresses: linianguang@njutcm.edu.cn (N.-G. Li), yupingtang@njutcm.edu.cn (Y.-P. Tang), dja@njutcm.edu.cn (J.-A. Duan).
[1] Co-first authors.

0223-5234/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2012.04.044

Because thrombin plays a pivotal role in thrombogenesis and in order to treat these thrombotic diseases, some thrombin inhibitors such as heparin, hirudin, bivalirudin and argatroban are used and have obtained great efficiency [1–3]. However, some side effects of these thrombin inhibitors have limited their clinical application, for example, heparin has been associated with very harmful side effects, including hypotension and intracranial bleeding and both can cause death [4].

In China, due to an increasing public interest in alternative medicine and disease prevention, traditional Chinese medicines (TCMs) containing rich flavonoids such as *Carthamus tinctorius* L. [5], *Abelmoschus manihot* L. [6], *Ginkgo biloba* L. [7] have been used in clinic to treat thrombotic diseases for many years, so natural products like flavonoids (e.g. flavones and flavanones) maybe promising lead compounds and studied as thrombin inhibitors.

In the previous research, we studied the thrombin inhibition activities of 30 natural flavonoids [8] including flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols and chalcones, and we found that quercetin (Fig. 1) was the strongest thrombin inhibitor tested.

Preliminary SARs analysis showed that a hydroxyl group at C-3 position played a key role in the inhibitory activity and more OH groups in the B-ring could increase thrombin inhibition activity. Furthermore, it was found that the presence of C(2)=C(3) and C(4)=O bonds were very important in showing the activity. It was also important to note that the presence of glycosyl groups at C-3 position greatly reduced the thrombin inhibition activity.

In blood, quercetin was mainly found in metabolized forms involving three main modifications on the phenolic hydroxyl groups: methylation, sulfation and glucuronidation [9]. Analysis of plasma from pigs fed with quercetin-rich diets showed that quercetin was absent and only methylated metabolites, such as the 3′-*O*-methylquercetin (isorhamnetin) (**2**) and the 4′-*O*-methylquercetin (tamarixetin) (**3**) (Fig. 2), mainly conjugated as glucuronides or sulfates [10], were present. With some variations in the relative abundance of the methylation positions, the same metabolism was also observed in rat [11] and *in vivo* cell culture [12]. Because the glucuronide or sulfate groups are readily deconjugated in tissues [13], these methylated metabolites are presumably the active molecules. Some studies using plasma samples contained sulfate or glucuronide conjugates or both and methylated forms showed that the activities of these metabolites were rather different from that of quercetin [14].

In order to study the cardiovascular activities of methylated quercetin metabolites and other methylated quercetin, in the present paper, we report here on the synthesis of a series of monomethylated quercetin, dimethylated quercetin, trimethylated quercetin, tetramethylated quercetin and pentamethylated quercetin using the synthetic route we developed in our previous study [15]. Then the evaluation of these compounds in anticoagulant activities as thrombin inhibitors through the analyzation of prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB) are described. In addition, the Structure-activity relationship (SAR) analysis of these methylated quercetins as thrombin inhibitors are also discussed.

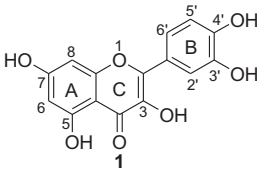


Fig. 1. Chemical structure of quercetin.

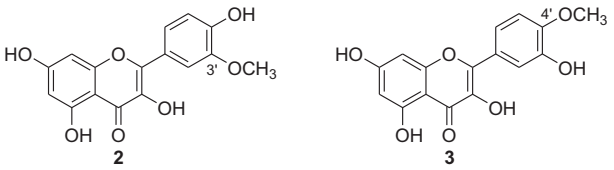


Fig. 2. Chemical structure of isorhamnetin (**2**) and tamarixetin (**3**).

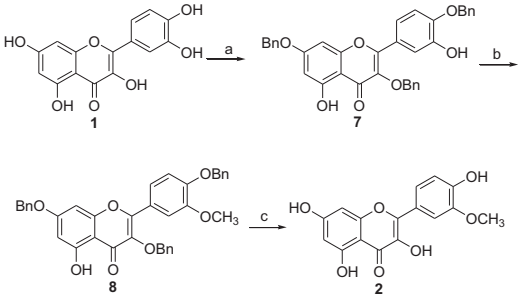
2. Results and discussion

2.1. Synthesis

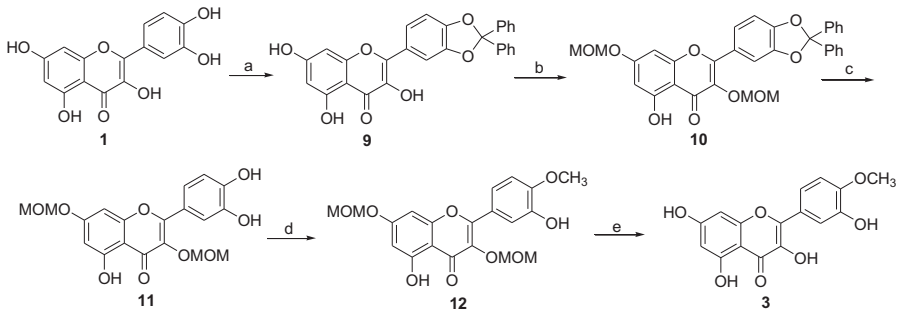
2.1.1. Synthesis of monomethylated quercetin

Although Rolando's group have synthesized all the five *O*-monomethylated analogs of quercetin in 2002 [16], however, the yields of some *O*-monomethylated quercetin were very low and the biological activities of these *O*-monomethylated analogs of quercetin have not been reported ever. So in this paper, we optimize the chemical synthetic routes in order to improve the yields of the target compounds. Preparation of 3′-*O*-methylquercetin **2** is shown in Scheme 1. Based on the different reactivity of five hydroxyl groups in quercetin following a specific sequential positions order: 4′ > 7 > 3 > 3′>5, the benzylation of quercetin with 3.0 equiv. of benzyl bromide led mainly to the formation of tribenzylated product **7**, however, Rolando's group obtained tribenzylated product **7** along with tetrabenzylquercetin and pentabenzylquercetin when 3.5 equiv. of benzyl bromide was used in this benzylation. Then partial methylation of the free phenolic function at C-3′ position in **7** with 1.0 equiv. of iodomethane (the C-5 position is less reactive) afforded **8** in 92% yield, finally, deprotection of the benzyl groups gave 3′-*O*-methylquercetin **2** in 90% yield.

The 4′-*O*-methylquercetin (tamarixetin) (**3**) is synthesized as summarized in Scheme 2. This route was based on hemisynthesis starting from quercetin (**1**) and relied on successive and selective protections of the different phenolic functions in quercetin developed by us [15]. Treatment of **1** with 1.5 equiv. of dichlorodiphenylmethane in diphenyl ether at 175 °C [17] afforded the desired product **9** in 86% yield after the reaction mixture had proceeded 30 min. Subsequently, reaction of **9** with an excess of chloromethyl ether (4.0 equiv.) and K_2CO_3 (4.2 equiv.) in acetone led to the formation of **10**, whose all phenolic functions except the hydroxyl group at C-5 position were protected. Fortunately, under hydrogen conditions the diphenylmethylene ketal can be cleaved selectively. The best results were obtained using 10% palladium on carbon as a catalyst in THF/EtOH, this method allowed deprotection to afford **11** in 95% yield and no side products were detected by TLC analysis. Treatment of **11** with 1.2 equiv. of iodomethane led selectively to **12** with a methyl group at the C-4′ position in 92% yield. The ultimate step of this synthesis consisted of the hydrogenolysis of the methyl ether group catalyzed by hydrochloric acid,



Scheme 1. Reagents and conditions: (a) BnBr, K_2CO_3, DMF, 25 °C, 12 h, 80%; (b) MeI, K_2CO_3, DMF, 25 °C, 12 h, 92%; (c) Pd/C (10 wt%), H_2 (1 atm), EtOH, 25 °C, 8 h, 90%.

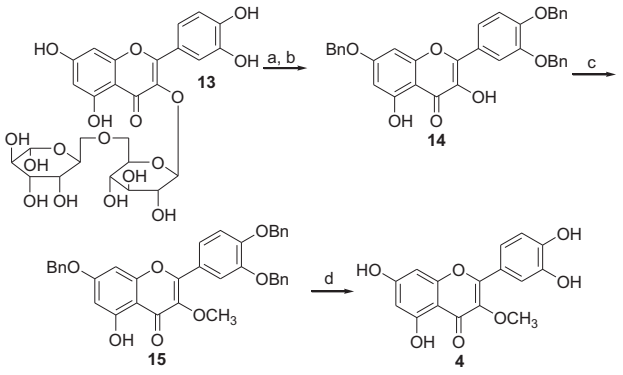


Scheme 2. Reagents and conditions: (a) Ph_2CCl_2, Ph_2O, 175 °C, 30 min, 86%; (b) MOMCl, K_2CO_3, acetone, reflux, 6 h, 93%; (c) Pd/C (10 wt%), H_2 (1 atm), THF/EtOH, 8 h, 95%; (d) MeI, K_2CO_3, DMF, 8 h, 92%; (e) HCl (1.0 M) in Et_2O/CH_2Cl_2 (1:1), 25 °C, 6 h, 90%.

which provided the 4′-*O*-methylquercetin (tamarixetin) **3** in 90% yield.

The 3-*O*-methylquercetin (**4**) [18] is synthesized as summarized in Scheme 3. Selective benzylation of rutin with 3.0 equiv. of benzyl bromide and hydrolysis of glycosidic bond with HCl led mainly to the formation of tribenzylated product **14**, then regioselective methylation of 3-OH with 5-OH unreacted afforded **15** in 95% yield, at last cleavage of the benzyl group by hydrogenolysis gave 3-*O*-methylquercetin **4** in 91% yield.

5-*O*-Methylquercetin (**5**) and 7-*O*-Methylquercetin (**6**) are synthesized as shown in Scheme 4. Treatment of **9** with 2.0 equiv. of benzyl bromide in the presence of K_2CO_3 afforded two products: the 3,7-dibenzyl isomer (**16**) and the 3-benzyl isomer (**17**) which were easily separated by chromatography on silica gel. Treatment of **16** with 5.0 equiv. of iodomethane led to **18** in 94% yield, under hydrogenation conditions diphenylmethylene ketal and benzyl groups were deprotected with 10% palladium on carbon as the catalyst in THF/EtOH afforded **5** in 92% yield. Treatment of **17** with 1.2 equiv. of iodomethane led selectively to **19** in 93% yield with the desired methyl group at C-7 position, then the deprotection of diphenylmethylene ketal and benzyl groups under hydrogenation conditions using 10% palladium on carbon as the catalyst in THF/EtOH afforded **6** in 93% yield.

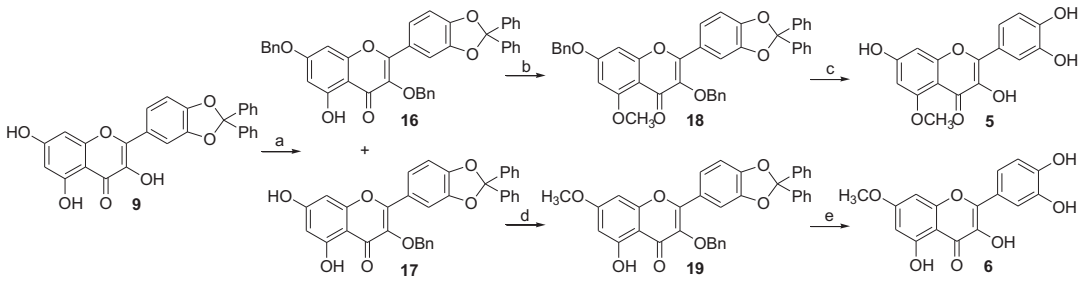


Scheme 3. Reagents and conditions: (a) BnBr, K_2CO_3, DMF, 40 °C, 3 h; (b) 36.5% HCl, 95% EtOH, 70 °C, 2 h, 92% over 2 steps; (c) MeI, K_2CO_3, DMF, 8 h, 95%; (d) Pd/C (10 wt%), H_2 (1 atm), EtOH/THF, 25 °C, 8 h, 91%.

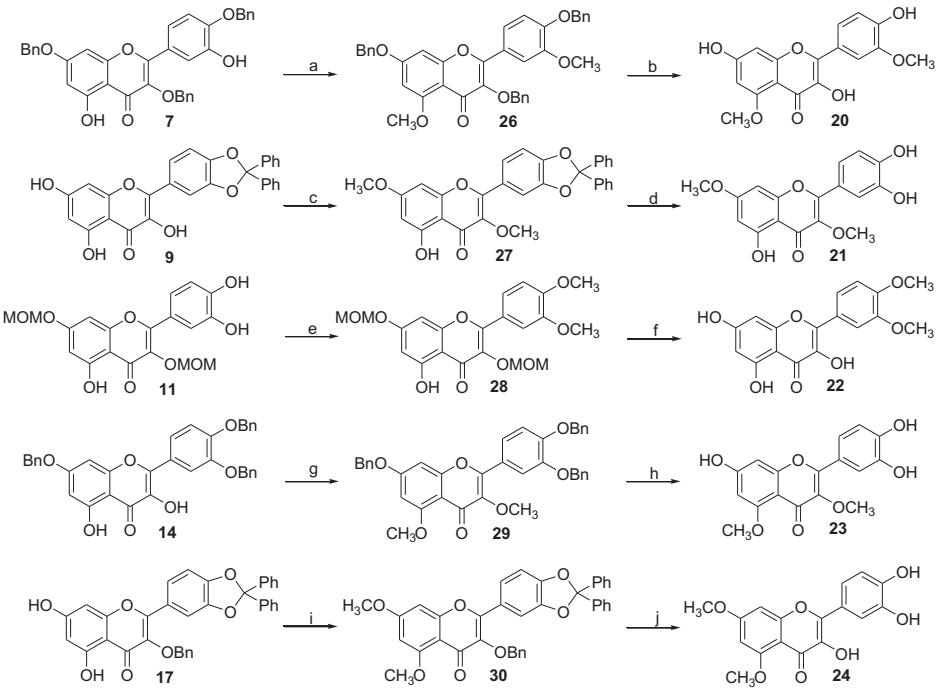
2.1.2. Synthesis of multimethylated quercetin

After we have optimized the synthetic routes to the construction of the monomethylated quercetins, we used these synthetic routes to the synthesis of the multimethylated quercetins.

The synthesis of the dimethylated quercetins is shown in Scheme 5. Methylation of the free phenolic function at C-3′ and C-5 positions in tribenzylated quercetin **7** with an excess of iodomethane gave **26**, then the cleavage of the benzyl groups by hydrogenolysis on 10% palladium on carbon in THF/EtOH (1:1) mixture at room temperature afforded **20**. Treatment of **9** with 2.6 equiv. of iodomethane in the presence of K_2CO_3 afforded **27**, then the 3,7-*O*-dimethylquercetin **21** was directly obtained in 93% yield after hydrogenolysis of the diphenylmethylene ketal at C-3′ and C-4′ positions using 10% palladium on carbon in THF/EtOH (1:1) mixture at room temperature. Compound **11** reacted with 2.6 equiv. of iodomethane led to **28** with the two methyl group at C-3′ and C-4′ positions in 92% yield, then the hydrolysis of the methyl ether group with hydrochloric acid gave **22** in 91% yield. Methylation of the free phenolic function at C-3 and C-5 positions in **14** with an excess of iodomethane and K_2CO_3 gave **29** in 91%, and the deprotection step involving the cleavage of the benzyl groups by hydrogenolysis on 10% palladium on carbon in THF/EtOH (1:1) at room temperature afforded **23** in 91% yield. During the methylation of the phenolic function at C-5 and C-7 positions in **17**, an excess of iodomethane and K_2CO_3 were used to give **30**, then the deprotection of the two kinds of protecting group by hydrogenolysis on 10% palladium on carbon in THF/EtOH (1:1) mixture at room temperature afforded **24** in 92% yield.

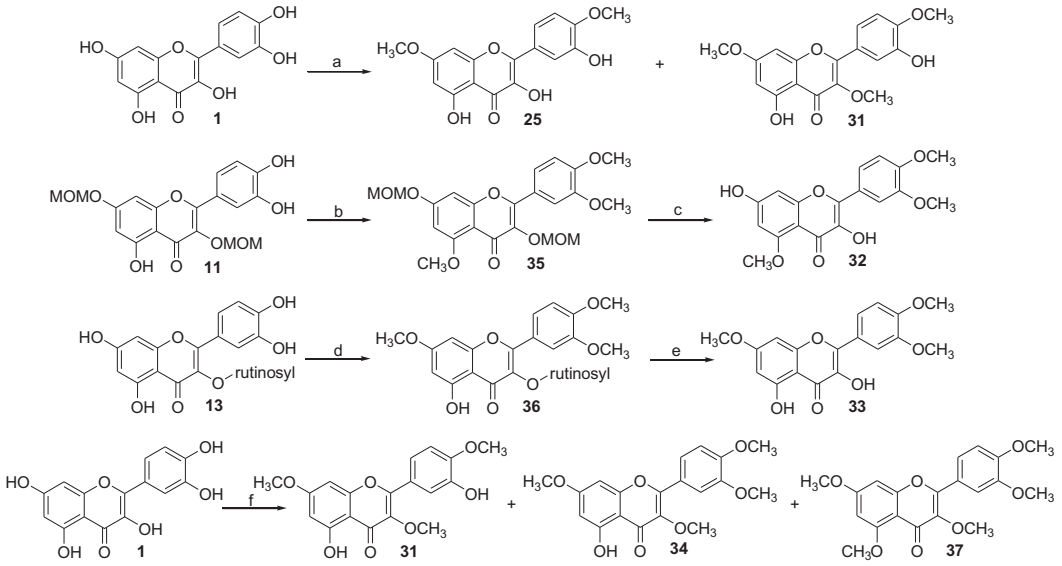


Scheme 4. Reagents and conditions: (a) BnBr, K_2CO_3, DMF, 25 °C, 12 h, **16** (24% isolated yield), **17** (63% isolated yield); (b) MeI, K_2CO_3, DMF, 25 °C, 12 h, 94%; (c) Pd/C (10 wt%), H_2 (1 atm), THF/EtOH, 12 h, 92%. (d) MeI, K_2CO_3, DMF, 25 °C, 12 h, 93%; (e) Pd/C (10 wt%), H_2 (1 atm), THF/EtOH, 12 h, 93%.



Scheme 5. Reagents and conditions: (a) MeI, K_2CO_3, DMF, 25 °C, 12 h, 93%; (b) Pd/C (10 wt%), H_2 (1 atm), THF/EtOH, 12 h, 92%. (c) MeI, K_2CO_3, DMF, 25 °C, 12 h, 94%; (d) Pd/C (10 wt%), H_2 (1 atm), THF/EtOH, 12 h, 93%. (e) MeI, K_2CO_3, DMF, 25 °C, 12 h, 92%; (f) HCl (1.0 M) in Et_2O/CH_2Cl_2 (1:1), 25 °C, 6 h, 91%; (g) MeI, K_2CO_3, DMF, 25 °C, 12 h, 91%; (h) Pd/C (10 wt%), H_2 (1 atm), THF/EtOH, 12 h, 91%; (i) MeI, K_2CO_3, DMF, 25 °C, 12 h, 92%; (j) Pd/C (10 wt%), H_2 (1 atm), THF/EtOH, 12 h, 92%.

The synthesis of the trimethylated quercetins, tetramethylated quercetins and pentamethylated quercetins is shown in Scheme 6. Treatment of **1** with 3.5 equiv. of iodomethane in the presence of K_2CO_3 afforded a mixture of two main products: the 4′,7-dimethyl isomer **25** and the 3,4′,7-trimethyl isomer **31** (Scheme 6) which were easily separated by chromatography on silica gel. The methyl group positions in **25** were confirmed by ROESY spectrum (Fig. 3), a cross-peak observed in the ROESY spectrum between δ 3.98 ($C_{4'}$–OCH_3) with 6.98 ($C_{5'}$–H) confirmed that the position of the methyl group was at $C_{4'}$–O, another cross-peak between δ 3.86 (C_7–OCH_3) with 6.28 (C_6–H) and 6.41 (C_8–H) confirmed that the position of the methyl group was at C_7–O (Fig. 3). The methyl group positions in **31** were confirmed by ^{1}H NMR spectrum and ROESY spectrum (Fig. 3), in the ^{1}H NMR spectrum, a signal at δ 12.63 confirmed that there was no methyl group at C_5–O position, a cross-peak observed in the ROESY spectrum between δ 3.99 ($C_{4'}$–OCH_3) with 6.96 ($C_{5'}$–H) confirmed that the position of the methyl group was at $C_{4'}$–O, another cross-peak between δ 3.87 (C_7–OCH_3) with 6.35 (C_6–H) and 6.44 (C_8–H) confirmed that the position of the methyl group was at C_7–O, no cross-peak between



Scheme 6. Reagents and conditions: (a) MeI, K_2CO_3, DMF, 25 °C, 12 h, **25** (28% isolated yield), **31** (60% isolated yield); (b) MeI, K_2CO_3, DMF, 25 °C, 12 h, 90%; (c) HCl (1.0 M) in Et_2O/CH_2Cl_2 (1:1), 25 °C, 6 h, 92%. (d) MeI, K_2CO_3, DMF, 40 °C, 3 h; (e) 36.5% HCl, 95% EtOH, 70 °C, 2 h, 91% over 2 steps; (f) MeI, K_2CO_3, DMF, 25 °C, 12 h, **31** (20% isolated yield), **34** (32% isolated yield), **37** (41% isolated yield).

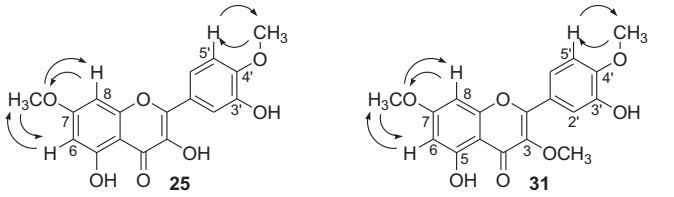


Fig. 3. ROESY correlations of dimethylated quercetin **25** and trimethylated quercetin **31**.

δ 3.87 (C_3—OCH_3) with 7.69 ($C_{2'}$—H) confirmed that the position of the methyl group was at C_3—O (Fig. 3).

Treatment of **11** with 6.0 equiv. of iodomethane led to the formation of **35** with the desired methyl groups at C-3′, C-4′ and C-5 positions in 90% yield. Then hydrolysis of the methyl ether group with hydrochloric acid gave **32** in 92% yield. The trimethylquercetin **33** was synthesized from commercially low-cost rutin **13** in two steps including methylation and hydrolysis of glycosidic bond. Treatment of **1** with 5.5 equiv. of iodomethane in the presence of K_2CO_3 afforded a mixture of three products: the trimethylated quercetin **31**, the tetramethylated quercetin **34** and the pentamethylated quercetin **37** (Scheme 6) which were easily separated by chromatography on silica gel.

2.2. Anti-thrombic activity

Because the thrombin inhibition activity can be assessed by assaying the prolongation of the plasma clotting time of TT, APTT, INR increasement of PT, and reduction of FIB content according to our previous studies [8], so the thrombin time of different compounds was investigated for TT, PT, APTT and FIB. The results were shown in Table 1.

In the series of monomethylated quercetins, the most active compounds were 5-*O*-methylquercetin (**5**) and 7-*O*-methylquercetin (**6**), these two compounds significantly prolonged TT and APTT, increased PT and decreased FIB content compared to quercetin. In 3′-*O*-methylquercetin (**2**) and 3-*O*-methylquercetin (**4**), TT and APTT were prolonged and FIB content decreased, however, PT decreased compared to quercetin. 4′-*O*-methylquercetin (**3**) showed no more active effect on plasma coagulation parameters than quercetin, despite it prolonged PT and APTT, but TT decreased and FIB content increased compared to quercetin.

Table 1
Effect of *O*-methylated analogs of quercetin on thrombin time (TT).

Compd. (100 μM)	Plasma coagulation parameters			
	TT (s)	PT (s)	APTT (s)	FIB (g/L)
1	19.2 ± 1.60	6.2 ± 0.17	30.3 ± 1.71	5.8 ± 0.03
2	20.5 ± 1.51	5.3 ± 0.23	82.3 ± 5.15	5.4 ± 0.11
3	16.6 ± 1.62	6.9 ± 0.27	49.8 ± 2.66	7.5 ± 0.08
4	22.9 ± 1.00	4.8 ± 0.23	50.2 ± 4.45	4.7 ± 0.04
5	19.4 ± 1.15	7.6 ± 0.64	49.2 ± 5.51	5.0 ± 0.10
6	23.0 ± 1.17	6.8 ± 0.38	72.6 ± 5.07	5.4 ± 0.18
20	22.3 ± 1.67	5.8 ± 0.13	57.6 ± 8.99	6.2 ± 0.09
21	22.5 ± 1.51	7.3 ± 0.28	54.7 ± 3.75	7.3 ± 0.05
22	9.9 ± 1.05	5.8 ± 0.36	58.7 ± 3.10	6.6 ± 0.03
23	20.0 ± 1.25	5.7 ± 0.58	63.7 ± 3.43	5.4 ± 0.02
24	21.8 ± 0.85	6.9 ± 0.48	67.6 ± 6.95	5.1 ± 0.52
25	14.8 ± 1.02	7.4 ± 0.76	46.2 ± 3.37	5.9 ± 0.04
31	14.9 ± 0.56	6.0 ± 0.28	61.7 ± 3.47	5.3 ± 0.07
32	15.9 ± 1.11	5.1 ± 0.39	49.3 ± 4.82	7.9 ± 0.01
33	13.4 ± 0.67	5.8 ± 0.22	52.2 ± 1.65	6.7 ± 0.01
34	14.2 ± 1.01	5.4 ± 0.27	42.4 ± 5.06	8.4 ± 0.04
37	11.6 ± 1.18	5.2 ± 0.60	40.3 ± 7.37	8.9 ± 0.08

Data represent mean ± S.D. $n = 4$.

In the series of dimethylated quercetins, the most active compound was **24**, it prolonged TT and APTT, increased PT and decreased FIB content significantly compared to quercetin. The less active compounds were **21** and **23**, compound **21** only increased FIB content compared to quercetin and **23** only decreased PT compared to quercetin. The least active compounds were **20** and **25**, for example, compound **20** increased TT and APTT, however, it decreased PT and increased FIB content compared to quercetin, compound **25** increased PT and APTT, however, it decreased TT and increased FIB content compared to quercetin. Compound **22** showed no better active than quercetin, although it increased APTT, however, it decreased TT and PT, and increased FIB content compared to quercetin.

Among the series of multimethlyated quercetins, there was no compound showed better anticoagulant activity than quercetin. However, compound **31** showed better anticoagulant activity in PT, APTT and FIB content than the other two trimethlyated quercetin derivatives **32** and **33**, with its PT was 6.0 s, its APTT was 61.7 s and its FIB content was 5.3 g/L. Tetramethylated quercetin **34** and pentamethylated quercetin **37** showed little effects on plasma coagulation parameter, and it only increased APTT compared to quercetin.

2.3. Docking studies

In the thrombin inhibition tests, 3-*O*-methylquercetin (**4**), 5-*O*-methylquercetin (**5**), 7-*O*-methylquercetin (**6**), 3,7-*O*-dimethylquercetin (**21**), 3,5-*O*-dimethylquercetin (**23**) and 5,7-*O*-dimethylquercetin (**24**) exhibited better inhibitory activities than quercetin. These six methylquercetin analogs and quercetin were selected for the subsequent molecular docking experiment.

There were three pockets (S1, S2, S3) in the thrombin (2R2M) as suggested by molecular modeling augured well for anticipating in vitro activity as well [19,20]. In the binding mode of the ligands and thrombin (2R2M), as shown in Fig. 4, ligand and the potent thrombin inhibitor **I-50** interacted with S1, S2, and S3 pockets, and the methylquercetin derivatives mainly interacted with S1 and S3 pocket.

As displayed in Fig. 5 and Table 2, ligand **I-50** formed eight hydrogen bonds with the active site residues of 2R2M in the binding mode, and the active site residues were Asp229(2), Ala230, Ser235, Ser256, Gly258(2), Gly260. Quercetin formed five hydrogen bonds with the active site residues Asp229(2), Ala230, Ser235, Gly258 of 2R2M, **4** formed three hydrogen bonds with the active site residues Tyr83, Ala230, Gly260 of 2R2M, **5** and **24** formed four hydrogen bonds with active site residues Asp229(2), Ser235, Gly258 of 2R2M respectively, **6** formed three hydrogen bonds with the active site residues Asp229, Gly258, Gly260 of 2R2M, **21** formed two hydrogen bonds with the active site residues Ala230, Gly258 of 2R2M, **23** formed three hydrogen bonds with the active site residues Asp229, Ala230, Gly258 of 2R2M.

2.4. Discussion and SAR

In this study, 17 quercetin derivatives were synthesized based on metabolism *in vivo* and were investigated for TT, PT, APTT and FIB. Quercetin (**1**), 3-*O*-methylquercetin (**4**), 5-*O*-methylquercetin (**5**), 7-*O*-methylquercetin (**6**), 3,7-*O*-dimethylquercetin (**21**), 3,5-*O*-dimethylquercetin (**23**) and 5,7-*O*-dimethylquercetin (**24**) were selected for docking experiments for the molecular modeling investigation.

Compounds **2**—**6** belonged to monomethylated quercetin, which had only one methyl group substituted on the quercetin nucleus. When OH groups in the B-ring or in the C-ring were replaced by OCH_3 groups, thrombin inhibition activities reduced. 3′-*O*-

methylquercetin (**2**), which had one OCH_3 group (C-3′ position) and one OH group (C-4′ position) in the B-ring, showed weaker thrombin inhibition activity compared with quercetin (**1**) (C-3′, C-4′ positions were OH groups) in PT. The same as 4′-*O*-methylquercetin (**3**) which had one OCH_3 group (C-4′ position) and one OH group (C-3′ position) in the B-ring, also showed weaker thrombin inhibition activity than quercetin (**1**) (C-3′, C-4′ positions were OH groups) in TT and FIB. 3-*O*-Methylquercetin (**4**), which had one OCH_3 group (C-3 position) in the C-ring, showed weaker thrombin inhibition activity than quercetin (**1**) (C-3′, C-4′ positions were OH groups) in PT. These results showed that the 3′-hydroxyl group and 4′-hydroxyl group in the B-ring and 3-hydroxyl group in the C-ring were very important for the thrombin inhibition activity. In addition, 3′-*O*-methylquercetin (**2**) and 3-*O*-methylquercetin (**4**) had stronger anticoagulant activity than 4′-*O*-methylquercetin (**3**), which meant that 4′-hydroxyl group was more important in

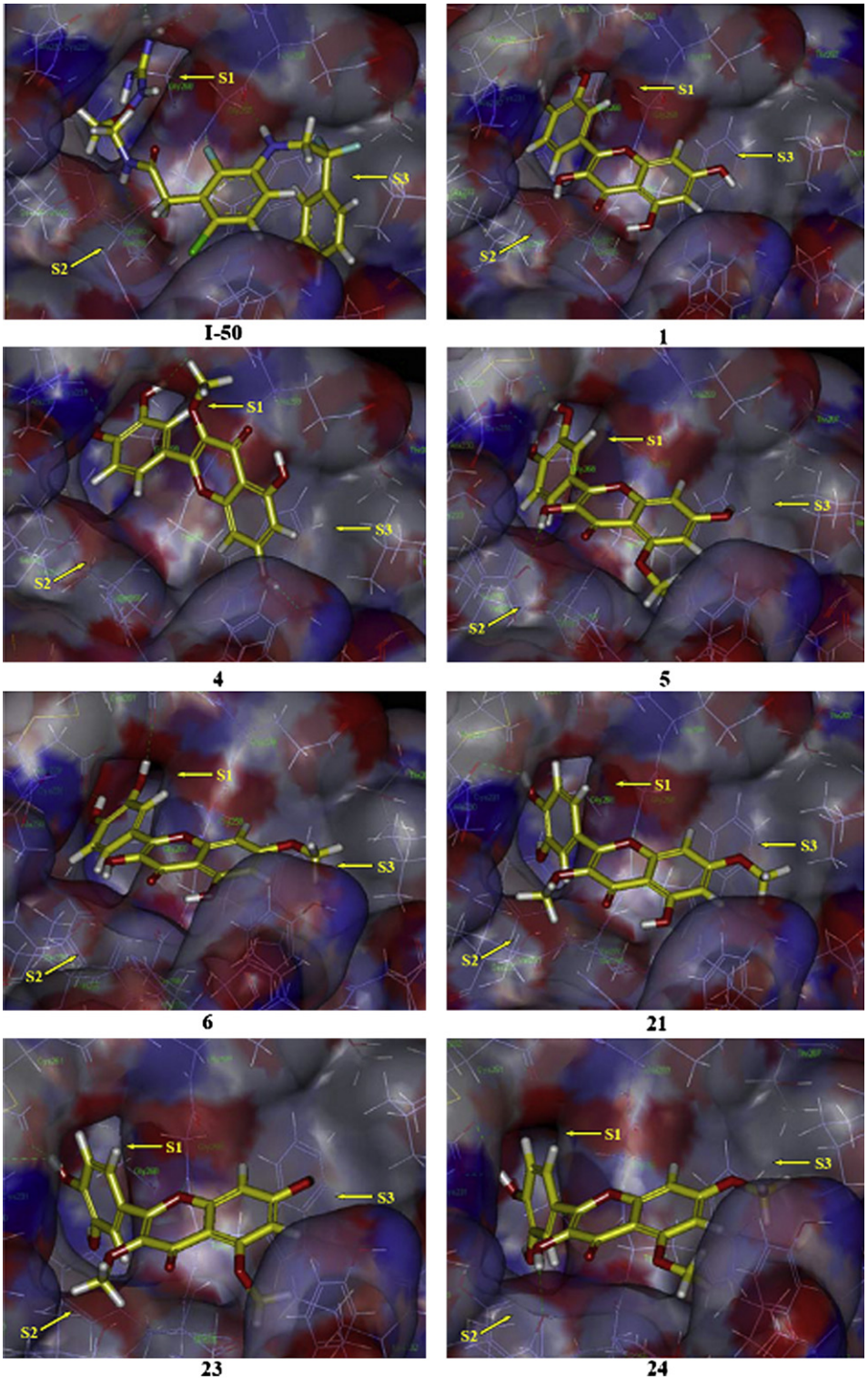


Fig. 4. I-50 and quercetin derivatives docked into the pockets of 2R2M.

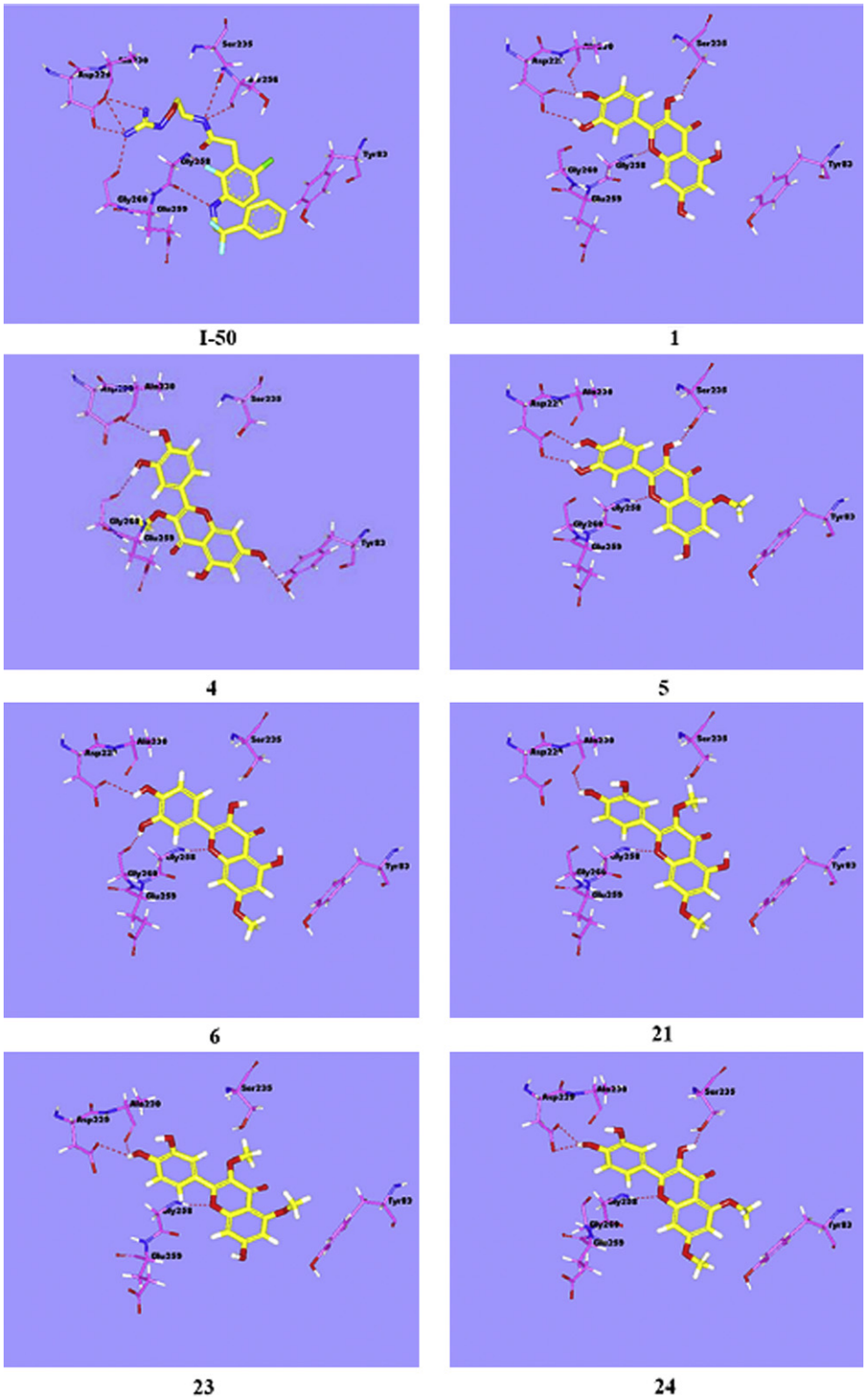


Fig. 5. I-50 and quercetin derivatives docked into residues of 2R2M, hydrogen bonding interactions are shown as red dashed lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

anticoagulant activity than 3′-hydroxyl group and 3-hydroxyl group. When OH group in the A-ring was replaced by OCH_3 groups, thrombin inhibition activity was improved. For example, 5-*O*-methylquercetin (**5**), which had one OCH_3 group (C-5 position) in the A-ring, had stronger thrombin inhibition activity compared with quercetin (**1**). The same as 7-*O*-methylquercetin (**6**), which had one OCH_3 group (C-7 position) in the A-ring, also had stronger thrombin inhibition activity than quercetin (**1**). These results indicated that 5-hydroxyl group and 7-hydroxyl group can be modified without reducing the anticoagulant activity.

In the series of dimethylated quercetin **20**–**25**, 3′,5-*O*-dimethylquercetin (**20**), which had one OCH_3 group (C-5 position) in the

Table 2
I-50 and quercetin derivatives formed hydrogen bonds with the active site residues.

Ligand	Number of hydrogen bonds	Hydrogen bonds residues
I-50	8	Asp229(2), Ala230, Ser235, Ser256, Gly258(2), Gly260.
1	5	Asp229(2), Ala230, Ser235, Gly258.
4	3	Tyr83, Ala230, Gly260.
5	4	Asp229(2), Ser235, Gly258.
6	3	Asp229, Gly258, Gly260.
21	2	Ala230, Gly258.
23	3	Asp229, Ala230, Gly258.
24	4	Asp229(2), Ser235, Gly258.

A-ring and one OCH_3 group (C-3′ position) in the B-ring, had weaker thrombin inhibition activity compared with quercetin (**1**) in PT and FIB, 3′,4′-*O*-dimethylquercetin (**22**), which had two OCH_3 groups (C-3′, C-4′ positions) in the B-ring, had weaker thrombin inhibition activity compared with quercetin (**1**) in TT, PT and FIB. Meanwhile, 3,7-*O*-dimethylquercetin (**21**), 3,5-*O*-dimethylquercetin (**23**) and 5,7-*O*-dimethylquercetin (**24**), which had two OH groups in the A-ring and C-ring, exhibited stronger thrombin inhibition activity than **20** and **22**, these results confirmed that the presence of OH groups (C-3′, C-4′ positions) in the B-ring were very important for the thrombin inhibition activity. In addition, 5,7-*O*-dimethylquercetin (**24**) exhibited most anticoagulant activity among the dimethylated quercetin derivatives. Furthermore, among the trimethylated quercetin **31**–**33**, tetramethylated quercetin **34** and pentamethylated quercetin **37**, only **31** had one OH group (C-3′ position) in the B-ring, it exhibited stronger thrombin inhibition activity than that of other multimethylated quercetin derivatives. All these results revealed that the presence of OH group (C-3′, C-4′ positions) in the B-ring and the presence of OH group (C-3 position) in the C-ring were very important in showing thrombin inhibition activity.

Quercetin (**1**), 3-*O*-methylquercetin (**4**), 5-*O*-methylquercetin (**5**), 7-*O*-methylquercetin (**6**), 3,7-*O*-dimethylquercetin (**21**), 3,5-*O*-dimethylquercetin (**23**) and 5,7-*O*-dimethylquercetin (**24**) were selected for docking experiments for the molecular modeling investigation. The results showed that the OH groups (C-3′, C-4′ positions) in the B-ring could form hydrogen bonds with the active site residue Asp229 and (or) Ala230 in the S1 pocket. Although 5-*O*-methylquercetin (**5**) and 5,7-*O*-dimethylquercetin (**24**) both formed four hydrogen bonds with active site residues, the thrombin inhibition activity of 5,7-*O*-dimethylquercetin (**24**) was stronger than that of 5-*O*-methylquercetin (**5**), this interesting phenomenon maybe due to that 7-OMe in **24** could interacted with more active site residues in the S3 pocket in the thrombin.

In this study, we examined the effects of 17 methylquercetin derivatives which were synthesized based on metabolism *in vivo* on thrombin inhibition activity. The results showed that 6 methylquercetin derivatives had stronger inhibitory activities than that of quercetin. They were as follows: 3-*O*-methylquercetin (**4**), 5-*O*-methylquercetin (**5**), 7-*O*-methylquercetin (**6**), 3,7-*O*-dimethylquercetin (**21**), 3,5-*O*-dimethylquercetin (**23**) and 5,7-*O*-dimethylquercetin (**24**). Preliminary SARs analysis showed that hydroxyl groups at C-3′ and C-4′ position in the B-ring played a key role in the thrombin inhibitory activity. Furthermore, it was found that the presence of hydroxyl group at C-3 position in the C-ring was very important in showing the activity. It was also important to note that the substitution of hydroxyl groups in B-ring and C-ring greatly reduced the thrombin inhibition activity. Therefore, the findings of this study would facilitate the design of chemical compounds with higher potency to serve as potential thrombin inhibitors, and provide information for the exploitation and utilization of quercetin as thrombin inhibitor for thrombotic disease treatment.

3. Experimental

3.1. 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 °C at approximately 20 mm Hg. All non-aqueous reactions were carried out under anhydrous conditions using flame-dried glassware within an argon atmosphere in dry, freshly distilled solvents, unless otherwise noted. Yields referred to chromatographically and spectroscopically (^{1}H NMR) 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. The melting points (Mp) were measured on a WRS-1B apparatus and were not corrected. ^{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.

3.1.1. 3,7-Bis(benzyloxy)-2-(4-(benzyloxy)-3-hydroxyphenyl)-5-hydroxy-4H-chromen-4-one (7)

To a solution of quercetin **1** (1.00 g, 2.96 mmol) in DMF (50 ml) was added K_2CO_3 (1.22 g, 8.87 mmol, 3.0 equiv.) and benzyl bromide (1.06 ml, 8.87 mmol, 3.0 equiv.). After vigorous 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. The resulting mixture was diluted with water (100 ml), extracted with ethyl acetate (100 ml), then the organic layer was washed with brine (100 ml), dried over $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (33% ethyl acetate in petroleum ether) to yield the tribenzylether **7** (1.35 g, 80% yield) as a yellow solid [16]. Mp 149–150 °C (lit. [16] 150–152 °C). ^{1}H NMR ($CDCl_3$, 500 MHz): 5.07 (s, 2H, $-OCH_2Ph$), 5.13 (s, 2H, $-OCH_2Ph$), 5.19 (s, 2H, $-OCH_2Ph$), 6.43 (d, J = 2.2 Hz, 1H, 6-H), 6.49 (d, J = 2.2 Hz, 1H, 8-H), 6.95 (d, J = 9.2 Hz, 1H, 5′-H), 7.21–7.47 (m, 15H, aromatic H), 7.60 (dd, J = 2.1, 9.2 Hz, 1H, 6′-H), 7.62 (d, J = 2.1 Hz, 1H, 2′-H), 12.68 (s, 1H, 5-OH). ESI-MS *m*/*z*: 573 $[M + H]^+$, 595 $[M + Na]^+$.

3.1.2. 3,7-Bis(benzyloxy)-2-(4-(benzyloxy)-3-methoxyphenyl)-5-hydroxy-4H-chromen-4-one (8)

To a stirring mixture of tribenzylether **7** (1.20 g, 2.10 mmol) and K_2CO_3 (377 mg, 2.73 mmol, 1.3 equiv.) in DMF (30 ml) was added methyl iodide (0.13 ml, 2.10 mmol, 1.0 equiv.) at room temperature, after 12 h, the resulting mixture was diluted with water (100 ml), extracted with ethyl acetate (100 ml), then the organic layer was washed with brine (100 ml), dried over $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to give **8** (1.23 g, 92% yield) as a yellow solid [16]. Mp 141–142 °C (lit. [16] mp 142–144 °C). ^{1}H NMR ($CDCl_3$, 500 MHz): 3.72 (s, 3H, $-OCH_3$), 5.07 (s, 2H, $-OCH_2Ph$), 5.13 (s, 2H, $-OCH_2Ph$), 5.23 (s, 2H, $-OCH_2Ph$), 6.44 (d, J = 2.2 Hz, 6-H), 6.50 (d, J = 2.2 Hz, 1H, 8-H), 6.94 (d, J = 9.2 Hz, 1H, 5′-H), 7.26–7.45 (m, 15H, aromatic H), 7.63 (dd,

J = 2.1, 9.2 Hz, 1H, 6′-H), 7.71 (d, J = 2.1 Hz, 1H, 2′-H), 12.68 (s, 1H, 5-OH). ESI-MS m/z: 587 $[M + H]^+$, 609 $[M + Na]^+$.

3.1.3. 3,5,7-Trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H-chromen-4-one (**2**)

To a solution of **8** (100 mg, 0.17 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 through celite and the filtrate concentrated. The crude material was then chromatographed over silica gel (50% ethyl acetate in petroleum ether) to yield **2** (48 mg, 90%) as a yellow solid [16]. Mp 302–304 °C (lit. [16] 301–303 °C). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.84 (s, 3H, $-OCH_3$), 6.19 (d, J = 2.0 Hz, 1H, 6-H), 6.48 (d, J = 2.0 Hz, 1H, 8-H), 6.94 (d, J = 8.4 Hz, 1H, 5′-H), 7.70 (dd, J = 1.8, 8.4 Hz, 1H, 6′-H), 7.75 (d, J = 1.8 Hz, 1H, 2′-H), 9.44 (s, 1H, 3-OH), 9.74 (s, 1H, 4′-OH), 10.77 (s, 1H, 7-OH), 12.47 (s, 1H, 5-OH). ESI-MS m/z: 317 $[M + H]^+$, 339 $[M + Na]^+$.

3.1.4. 2-(2,2-Diphenylbenzo[d][1,3]dioxol-5-yl)-3,5,7-trihydroxy-4H-chromen-4-one (**9**)

To a stirring mixture of quercetin (**1**) (302 mg, 1 mmol) in diphenyl ether (20 ml) was added dichlorodiphenylmethane (0.30 ml, 1.5 mmol, 1.5 equiv.), and the reaction mixture was heated at 175 °C for 30 min. The mixture was cooled to room temperature and the solid compound was obtained as the petroleum ether (50 ml) was added, then the solid was filtered, purified by column chromatography (25% ethyl acetate in petroleum ether) to yield **9** (400 mg, 86%) as a yellow solid [16]. Mp 218–219 °C (lit. [16] 222–224 °C). ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 6.20 (d, J = 2.0 Hz, 1H, 6-H), 6.47 (d, J = 2.0 Hz, 1H, 8-H), 7.22 (d, J = 8.8 Hz, 1H, 5′-H), 7.46 (m, 6H, aromatic H), 7.58 (m, 4H, aromatic H), 7.79 (dd, J = 1.8, 8.8 Hz, 1H, 6′-H), 7.82 (d, J = 1.8 Hz, 1H, 2′-H), 9.61 (s, 1H, 3-OH), 10.81 (s, 1H, 7-OH), 12.37 (s, 1H, 5-OH). ESI-MS m/z: 467 $[M + H]^+$, 489 $[M + Na]^+$.

3.1.5. 2-(2,2-Diphenylbenzo[d][1,3]dioxol-5-yl)-5-hydroxy-3,7-bis(methoxymethoxy)-4H-chromen-4-one (**10**)

To a stirring mixture of **9** (1.96 g, 4.21 mmol) and K_2CO_3 (2.44 g, 17.68 mmol, 4.2 equiv.) in dry acetone at room temperature was added chloromethyl ether (1.28 ml, 16.84 mmol, 4.0 equiv.). The reaction mixture was refluxed gently for 6 h. After cooling to the room temperature, the reaction mixture was filtered. Removal of the solvent under *vacuo* followed by silica gel column chromatographic purification of the residue using 20% ethyl acetate in petroleum ether afforded **10** (2.17 g, 93%) as a yellow solid. Mp 102–104 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 3.21 (s, 3H, $-OCH_3$), 3.48 (s, 3H, $-OCH_3$), 5.16 (s, 2H, $-OCH_2O-$), 5.22 (s, 2H, $-OCH_2O-$), 6.45 (d, J = 2.2 Hz, 1H, 6-H), 6.59 (d, J = 2.2 Hz, 1H, 8-H), 6.98 (d, J = 8.2 Hz, 1H, 5′-H), 7.39 (m, 6H, aromatic H), 7.59 (m, 5H, aromatic H), 7.65 (dd, J = 1.7, 8.2 Hz, 1H, 6′-H), 12.51 (s, 1H, 5-OH). ESI-MS m/z: 555 $[M + H]^+$, 577 $[M + Na]^+$.

3.1.6. 2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-bis(methoxymethoxy)-4H-chromen-4-one (**11**)

To a solution of **10** (100 mg, 0.18 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 through celite and the filtrate concentrated. The crude material was then chromatographed over silica gel (50% ethyl acetate in petroleum ether) to yield **11** (67 mg, 95%) as a yellow solid. Mp 142–143 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 3.18 (s, 3H, $-OCH_3$), 3.41 (s, 3H, $-OCH_3$), 5.12 (s, 2H, $-OCH_2O-$), 5.32 (s, 2H, $-OCH_2O-$), 6.44 (d, J = 2.2 Hz, 1H, 6-H), 6.75 (d, J = 2.2 Hz, 1H, 8-H), 6.91 (d, J = 8.4 Hz, 1H, 5′-H), 7.47 (dd, J = 2.4, 8.4 Hz, 1H, 6′-H), 7.54 (d, J = 2.4 Hz, 1H, 2′-H), 9.34 (s, 1H, 3′-OH), 9.76 (s, 1H, 4′-OH), 12.59 (s, 1H, 5-OH). ESI-MS m/z: 391 $[M + H]^+$, 413 $[M + Na]^+$.

3.1.7. 5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,7-bis(methoxymethoxy)-4H-chromen-4-one (**12**)

To a solution of **11** (100 mg, 0.26 mmol) in dry DMF (20 ml) was added K_2CO_3 (65 mg, 0.47 mmol, 1.8 equiv.) and iodomethane (0.019 ml, 0.31 mmol, 1.2 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 then washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield **12** (97 mg, 92%) as a yellow solid. Mp 136–138 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 3.25 (s, 3H, $-OCH_3$), 3.50 (s, 3H, $-OCH_3$), 3.98 (s, 3H, $-OCH_3$), 5.19 (s, 2H, $-OCH_2O-$), 5.23 (s, 2H, $-OCH_2O-$), 5.67 (s, 1H, 3′-OH), 6.45 (d, J = 2.1 Hz, 1H, 6-H), 6.61 (d, J = 2.1 Hz, 1H, 8-H), 6.96 (d, J = 9.2 Hz, 1H, 5′-H), 7.66 (dd, J = 2.0, 9.2 Hz, 1H, 6′-H), 7.68 (d, J = 2.0 Hz, 1H, 2′-H), 12.53 (s, 1H, 5-OH). ESI-MS m/z: 405 $[M + H]^+$, 427 $[M + Na]^+$.

3.1.8. 3,5,7-Trihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-chromen-4-one (**3**)

To a stirring solution **12** (404 mg, 1 mmol) of in CH_2Cl_2 (5 ml) and ethyl ether (5 ml) was added hydrochloric acid (1 ml) at 0 °C, the reaction mixture was allowed to warm to room temperature and stirred another 6 h. The reaction mixture was diluted with ethyl acetate (100 ml) and washed with water (100 ml) and brine (100 ml). The ethyl acetate layer was dried over $MgSO_4$, filtered and concentrated and the crude material was purified by column chromatography (50% ethyl acetate in petroleum ether) to yield **2** (284 mg, 90%) as a yellow solid [16]. Mp 253–255 °C (lit. [16] 252–254 °C). ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 3.85 (s, 3H, $-OCH_3$), 6.19 (d, J = 2.0 Hz, 1H, 6-H), 6.42 (d, J = 2.0 Hz, 1H, 8-H), 7.07 (d, J = 8.6 Hz, 1H, 5′-H), 7.64 (dd, J = 1.9, 8.6 Hz, 1H, 6′-H), 7.67 (d, J = 1.9 Hz, 1H, 2′-H), 9.29 (s, 1H, 3-OH), 9.40 (s, 1H, 3′-OH), 10.76 (s, 1H, 7-OH), 12.44 (s, 1H, 5-OH). ESI-MS m/z: 317 $[M + H]^+$, 339 $[M + Na]^+$.

3.1.9. 2-(3,4-Bis(benzyloxy)phenyl)-7-(benzyloxy)-3,5-dihydroxy-4H-chromen-4-one (**14**)

In order to take off the three crystal water, rutin (**13**) was dried under vacuum at 100 °C for 6 h. To a stirring mixture of rutin (**13**) (0.626 g, 1 mmol) and K_2CO_3 (0.58 g, 4.2 mmol, 4.2 equiv.) in DMF (30 ml) at room temperature was added benzyl bromide (0.42 ml, 3.5 mmol, 3.5 equiv.), then the reaction mixture was allowed to warm to 40 °C and the stirring was maintained for 3 h under argon. The resulting mixture was cooled to 0 °C and its pH was adjusted to 6 with 10% acetic acid, then the solid obtained was filtered, dissolved in 20 ml of 95% ethanol and reacted with 3 ml of 36% hydrochloric acid at 70 °C for 2 h. The reaction mixture was cooled to 0 °C, the solid obtained was filtered, washed with cold water and purified by column chromatography (25% ethyl acetate in petroleum ether) to give **14** (0.53 g, 92% yield) as a yellow solid [18]. Mp 190–191 °C (lit. [18] 188–190 °C). ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 5.21 (s, 2H, $-OCH_2Ph$), 5.25 (s, 4H, 2× $-OCH_2Ph$), 6.45(d, J = 2.2 Hz, 1H, 6-H), 6.86 (d, J = 2.2 Hz, 1H, 8-H), 7.26 (d, J = 8.6 Hz, 1H, 5′-H), 7.33–7.51 (m, 15H), 7.84 (dd, J = 2.2, 8.6 Hz, 1H, 6′-H), 7.89 (d, J = 2.2 Hz, 1H, 2′-H), 9.66 (s, 1H, 3-OH), 12.41 (s, 1H, 5-OH). ESI-MS m/z: 573 $[M + H]^+$, 595 $[M + Na]^+$.

3.1.10. 2-(3,4-Bis(benzyloxy)phenyl)-7-(benzyloxy)-5-hydroxy-3-methoxy-4H-chromen-4-one (**15**)

To a solution of **14** (149 mg, 0.26 mmol) in dry DMF (20 ml) was added K_2CO_3 (65 mg, 0.47 mmol, 1.8 equiv.) and iodomethane (0.019 ml, 0.31 mmol, 1.2 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 then washed with brine (100 ml), dried over $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield **15** (145 mg, 95%) as a yellow solid [18]. Mp 94–95 °C (lit. [18] 95–97 °C). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 3.71 (s, 3H, $-OCH_3$), 5.13 (s, 2H, $-OCH_2Ph$), 5.25 (s, 2H, $-OCH_2Ph$), 5.27 (s, 2H, $-OCH_2Ph$), 6.43 (d, J = 2.2 Hz, 1H, 6-H), 6.46 (d, J = 2.2 Hz, 1H, 6-H), 7.03 (d, J = 8.6 Hz, 1H, 5′-H), 7.31–7.49 (m, 15H), 7.65 (dd, J = 2.2, 8.6 Hz, 1H, 6′-H), 7.75 (d, J = 2.2 Hz, 1H, 2′-H), 12.61 (s, 1H, 5-OH). ESI-MS m/z: 587 $[M + H]^+$, 609 $[M + Na]^+$.

3.1.11. 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-4H-chromen-4-one (**4**)

To a solution of **15** (100 mg, 0.17 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 through celite and the filtrate concentrated. The crude material was then chromatographed over silica gel (50% ethyl acetate in petroleum ether) to yield **4** (49 mg, 91%) as a yellow solid [16,18]. Mp 273–274 °C (lit. [16] 271–273 °C, lit. [18] 273–275 °C). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.78 (s, 3H, $-OCH_3$), 6.19 (d, J = 1.8 Hz, 1H, 6-H), 6.41 (d, J = 1.8 Hz, 1H, 8-H), 6.90 (d, J = 8.4 Hz, 1H, 5′-H), 7.44 (dd, J = 1.8, 8.4 Hz, 1H, 6′-H), 7.54 (d, J = 1.8 Hz, 1H, 2′-H), 9.35 (s, 1H, 3′-OH), 9.72 (s, 1H, 4′-OH), 10.80 (s, 1H, 7-OH), 12.69 (s, 1H, 5-OH). ESI-MS m/z: 317 $[M + H]^+$, 339 $[M + Na]^+$.

3.1.12. Synthesis of dibenzylether **16** and monobenzylether **17**

To a solution of **9** (1.00 g, 2.15 mmol) in dry DMF (20 ml) was added K_2CO_3 (0.66 g, 4.73 mmol, 2.2 equiv.) and benzyl bromide (0.52 ml, 4.3 mmol, 2.0 equiv.) at 0 °C, then the mixture was warmed to 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 $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield dibenzylether **16** (0.33 g, 24% yield) and monobenzylether **17** (0.75 g, 63% yield) as yellow solids [16].

3.1.13. 3,7-Bis(benzyloxy)-2-(2,2-diphenylbenzo[d][1,3]dioxol-5-yl)-5-hydroxy-4H-chromen-4-one (**16**)

Mp 90–91 °C (lit. [16] 90–92 °C). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.03 (s, 2H, $-OCH_2Ph$), 5.11 (s, 2H, $-OCH_2Ph$), 6.43 (d, J = 2.1 Hz, 1H, 6-H), 6.46 (d, J = 2.1 Hz, 1H, 8-H), 6.90 (d, J = 8.6 Hz, 1H, 5′-H), 7.16–7.49 (m, 16H, aromatic H), 7.58 (dd, J = 2.1, 8.6 Hz, 1H, 6′-H), 7.60 (d, J = 2.1 Hz, 1H, 2′-H), 7.65–7.72 (m, 4H, aromatic H), 12.67 (s, 1H, 5-OH). ESI-MS m/z: 647 $[M + H]^+$, 669 $[M + Na]^+$.

3.1.14. 3-(Benzyloxy)-2-(2,2-diphenylbenzo[d][1,3]dioxol-5-yl)-5,7-dihydroxy-4H-chromen-4-one (**17**)

Mp 174–176 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.03 (s, 2H, $-OCH_2Ph$), 6.27 (d, J = 2.5 Hz, 1H, 6-H), 6.36 (d, J = 2.5 Hz, 1H, 8-H), 6.90 (d, J = 8.3 Hz, 1H, 5′-H), 7.13–7.20 (m, 5H, aromatic H), 7.27 (s, 1H, 7-OH), 7.39–7.43 (m, 6H, aromatic H), 7.49 (d, J = 1.8 Hz, 1H, 2′-H), 7.55 (dd, J = 8.3, 1.8 Hz, 1H, 6′-H), 7.58–7.60 (m, 4H, aromatic H), 12.74 (s, 1H, 5-OH). ESI-MS m/z: 557 $[M + H]^+$, 579 $[M + Na]^+$.

3.1.15. 3,7-Bis(benzyloxy)-2-(2,2-diphenylbenzo[d][1,3]dioxol-5-yl)-5-methoxy-4H-chromen-4-one (**18**)

To a solution of **16** (100 mg, 0.15 mmol) in dry DMF (20 ml) was added K_2CO_3 (1.24 g, 0.9 mmol, 6 equiv.) and iodomethane (0.047 ml, 0.75 mmol, 5 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 $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield **18** (93 mg, 94% yield) as a yellow solid. Mp 112–113 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.97 (s, 3H, $-OCH_3$), 5.05 (s, 2H, $-OCH_2Ph$), 5.13 (s, 2H, $-OCH_2Ph$), 6.44 (d, J = 2.2 Hz, 1H, 6-H), 6.55 (d, J = 2.2 Hz, 1H, 8-H), 6.89 (d, J = 8.2 Hz, 1H, 5′-H), 7.16–7.18 (m, 3H, aromatic H), 7.33–7.58 (m, 22H, aromatic H). ESI-MS m/z: 661 $[M + H]^+$, 683 $[M + Na]^+$.

3.1.16. 3-(Benzyloxy)-2-(2,2-diphenylbenzo[d][1,3]dioxol-5-yl)-5-hydroxy-7-methoxy-4H-chromen-4-one (**19**)

To a solution of **17** (100 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 $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield **19** (95 mg, 93% yield) as a yellow solid [16]. Mp 132–133 °C (lit. [16] 134–136 °C). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.86 (s, 3H, $-OCH_3$), 5.04 (s, 2H, $-OCH_2Ph$), 6.36 (d, J = 2.4 Hz, 1H, 6-H), 6.40 (d, J = 2.4 Hz, 1H, 8-H), 6.91 (d, J = 8.2 Hz, 1H, 5′-H), 7.15–7.20 (m, 5H, aromatic H), 7.40–7.44 (m, 6H, aromatic H), 7.50–7.62 (m, 6H, aromatic H), 12.68 (s, 1H, 5-OH). ESI-MS m/z: 571 $[M + H]^+$, 593 $[M + Na]^+$.

3.1.17. 2-(3,4-Dihydroxyphenyl)-3,7-dihydroxy-5-methoxy-4H-chromen-4-one (**5**)

To a solution of **18** (100 mg, 0.15 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 12 h, the reaction mixture was filtered through celite and the filtrate concentrated. The crude material was then chromatographed over silica gel (50% ethyl acetate in petroleum ether) to yield **5** (44 mg, 92%) as a yellow solid [16]. Mp 259–260 °C (lit. [16] 259–261 °C). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.83 (s, 3H, $-OCH_3$), 6.35 (d, J = 2.2 Hz, 1H, 6-H), 6.45 (d, J = 2.2 Hz, 1H, 8-H), 6.86 (d, J = 8.4 Hz, 1H, 5′-H), 7.48 (dd, J = 2.2, 8.4 Hz, 1H, 6′-H), 7.62 (d, J = 2.2 Hz, 1H, 2′-H), 8.61 (s, 1H, 3-OH), 9.24 (s, 1H, 3′-OH), 9.44 (s, 1H, 4′-OH), 10.69 (s, 1H, 7-OH). ESI-MS m/z: 317 $[M + H]^+$, 339 $[M + Na]^+$.

3.1.18. 2-(3,4-Dihydroxyphenyl)-3,5-dihydroxy-7-methoxy-4H-chromen-4-one (**6**)

To a solution of **19** (100 mg, 0.18 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 12 h, the reaction mixture was filtered through celite and the filtrate concentrated. The crude material was then chromatographed over silica gel (50% ethyl acetate in petroleum ether) to yield **6** (53 mg, 93%) as a yellow solid [16]. Mp 284–285 °C (lit. [16] 283–285 °C). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.87 (s, 3H, $-OCH_3$), 6.36 (d, J = 2.0 Hz, 1H, 6-H), 6.71 (d, J = 2.0 Hz, 1H, 8-H), 6.89 (d, J = 8.4 Hz, 1H, 5′-H), 7.58 (dd, J = 2.2, 8.4 Hz, 1H, 6′-H), 7.73 (d, J = 2.2 Hz, 1H, 2′-H), 9.30 (s, 1H, 3-OH), 9.50 (s, 1H, 3′-OH), 9.64 (s, 1H, 4′-OH), 12.49 (s, 1H, 5-OH). ESI-MS m/z: 317 $[M + H]^+$, 339 $[M + Na]^+$.

3.1.19. 3,7-Bis(benzyloxy)-2-(4-(benzyloxy)-3-methoxyphenyl)-5-methoxy-4H-chromen-4-one (**26**)

To a solution of **7** (286 mg, 0.5 mmol) in dry DMF (20 ml) was added K_2CO_3 (414 mg, 3.0 mmol, 6.0 equiv.) and iodomethane (0.16 ml, 2.5 mmol, 5.0 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 $MgSO_4$, filtered and concentrated.

The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield **26** (279 mg, 93% yield) as a yellow solid. Mp 122–123 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.70 (s, 3H, $-OCH_3$), 3.96 (s, 3H, $-OCH_3$), 5.07 (s, 2H, $-OCH_2Ph$), 5.13 (s, 2H, $-OCH_2Ph$), 5.23 (s, 2H, $-OCH_2Ph$), 6.43 (d, J = 2.2 Hz, 1H, 6-H), 6.56 (d, J = 2.2 Hz, 1H, 8-H), 6.92 (d, J = 8.6 Hz, 1H, 5′-H), 7.22–7.46 (m, 15H, aromatic H), 7.52 (dd, J = 2.1, 8.6 Hz, 1H, 6′-H), 7.68 (d, J = 2.1 Hz, 1H, 2′-H). ESI-MS m/z: 601 $[M + H]^+$, 623 $[M + Na]^+$.

3.1.20. 3,7-Dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-5-methoxy-4H-chromen-4-one (**20**)

To a solution of **26** (100 mg, 0.17 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 12 h, the reaction mixture was filtered through celite and the filtrate concentrated. The crude material was then chromatographed over silica gel (50% ethyl acetate in petroleum ether) to yield **20** (52 mg, 92%) as a yellow solid. Mp 240–241 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.84 (s, 3H, $-OCH_3$), 3.88 (s, 3H, $-OCH_3$), 6.36 (d, J = 2.0 Hz, 1H, 6-H), 6.55 (d, J = 2.0 Hz, 1H, 8-H), 6.94 (d, J = 8.4 Hz, 1H, 5′-H), 7.45 (dd, J = 1.8, 8.4 Hz, 1H, 6′-H), 7.51 (d, J = 1.8 Hz, 1H, 2′-H), 8.77 (s, 1H, 3-OH), 9.57 (s, 1H, 4′-OH), 10.69 (s, 1H, 7-OH). ESI-MS m/z: 331 $[M + H]^+$, 353 $[M + Na]^+$.

3.1.21. 2-(2,2-Diphenylbenzo[d][1,3]dioxol-5-yl)-5-hydroxy-3,7-dimethoxy-4H-chromen-4-one (**27**)

To a solution of **9** (233 mg, 0.5 mmol) in dry DMF (20 ml) was added K_2CO_3 (2.06 g, 1.5 mmol, 3.0 equiv.) and iodomethane (0.083 ml, 1.3 mmol, 2.6 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 $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield **27** (232 mg, 94% yield) as a yellow solid [16]. Mp 150–151 °C (lit. [16] 149–151 °C). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.86 (s, 6H, 2× $-OCH_3$), 6.34 (d, J = 2.0 Hz, 1H, 6-H), 6.42 (d, J = 2.0 Hz, 1H, 8-H), 7.00 (d, J = 8.3 Hz, 1H, 5′-H), 7.39 (m, 6H, aromatic H), 7.60 (m, 4H, aromatic H), 7.66 (d, J = 1.8 Hz, 1H, 2′-H), 7.70 (dd, J = 1.8, 8.3 Hz, 1H, 6′-H), 12.61 (s, 1H, 5-OH). ESI-MS m/z: 495 $[M + H]^+$, 517 $[M + Na]^+$.

3.1.22. 2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-4H-chromen-4-one (**21**)

To a solution of **27** (100 mg, 0.20 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 12 h, the reaction mixture was filtered through celite and the filtrate concentrated. The crude material was then chromatographed over silica gel (50% ethyl acetate in petroleum ether) to yield **21** (61 mg, 93%) as a yellow solid. Mp 229–230 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.80 (s, 3H, $-OCH_3$), 3.87 (s, 3H, $-OCH_3$), 6.37 (d, J = 2.0 Hz, 1H, 6-H), 6.71 (d, J = 2.0 Hz, 1H, 8-H), 6.91 (d, J = 8.4 Hz, 1H, 5′-H), 7.48 (dd, J = 1.8, 8.4 Hz, 1H, 6′-H), 7.59 (d, J = 1.8 Hz, 1H, 2′-H), 9.35 (s, 1H, 3′-OH), 9.77 (s, 1H, 4′-OH), 12.69 (s, 1H, 5-OH). ESI-MS m/z: 331 $[M + H]^+$, 353 $[M + Na]^+$.

3.1.23. 2-(3,4-Dimethoxyphenyl)-5-hydroxy-3,7-bis(methoxymethoxy)-4H-chromen-4-one (**28**)

To a solution of **11** (195 mg, 0.5 mmol) in dry DMF (20 ml) was added K_2CO_3 (2.06 g, 1.5 mmol, 3.0 equiv.) and iodomethane (0.083 ml, 1.3 mmol, 2.6 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 $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield **28** (192 mg, 92% yield) as a yellow solid. Mp 102–103 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.24 (s, 3H, $-OCH_3$), 3.52 (s, 3H, $-OCH_3$), 3.99 (s, 6H, 2× $-OCH_3$), 5.20 (s, 2H, $-OCH_2O-$), 5.26 (s, 2H, $-OCH_2O-$), 6.48 (d, J = 2.0 Hz, 1H, 6-H), 6.65 (d, J = 2.0 Hz, 1H, 8-H), 7.00 (d, J = 8.4 Hz, 1H, 5′-H), 7.66 (d, J = 2.0 Hz, 1H, 2′-H), 7.71 (dd, J = 2.0, 8.4 Hz, 1H, 6′-H), 12.57 (s, 1H, 5-OH). ESI-MS m/z: 419 $[M + H]^+$, 441 $[M + Na]^+$.

3.1.24. 2-(3,4-Dimethoxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (**22**)

To a stirring solution **28** (418 mg, 1 mmol) of in CH_2Cl_2 (5 ml) and ethyl ether (5 ml) was added hydrochloric acid (1 ml) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred another 6 h, then the reaction mixture was diluted with a large amount of ethyl acetate and washed with water and brine. The ethyl acetate layer was dried over $MgSO_4$, filtered and concentrated and the crude material was purified by column chromatography (50% ethyl acetate in petroleum ether) to yield **22** (300 mg, 91%) as a yellow solid. Mp 211–212 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.84 (s, 3H, $-OCH_3$), 3.85 (s, 3H, $-OCH_3$), 6.20 (d, J = 2.0 Hz, 1H, 6-H), 6.50 (d, J = 2.0 Hz, 1H, 8-H), 7.14 (d, J = 8.6 Hz, 1H, 5′-H), 7.75 (d, J = 2.0 Hz, 1H, 2′-H), 7.80 (dd, J = 2.0, 8.6 Hz, 1H, 6′-H), 9.54 (s, 1H, 3-OH), 10.79 (s, 1H, 7-OH), 12.43 (s, 1H, 5-OH). ESI-MS m/z: 331 $[M + H]^+$, 353 $[M + Na]^+$.

3.1.25. 2-(3,4-Bis(benzyloxy)phenyl)-7-(benzyloxy)-3,5-dimethoxy-4H-chromen-4-one (**29**)

To a solution of **14** (286 mg, 0.5 mmol) in dry DMF (20 ml) was added K_2CO_3 (414 mg, 3.0 mmol, 6.0 equiv.) and iodomethane (0.16 ml, 2.5 mmol, 5.0 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 $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield **29** (273 mg, 91% yield) as a yellow solid. Mp 132–134 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.74 (s, 3H, $-OCH_3$), 3.94 (s, 3H, $-OCH_3$), 5.13 (s, 2H, $-OCH_2Ph$), 5.25 (s, 4H, 2×$-OCH_2Ph$), 6.41 (d, J = 2.4 Hz, 1H, 6-H), 6.52 (d, J = 2.4 Hz, 1H, 8-H), 7.02 (d, J = 8.6 Hz, 1H, 5′-H), 7.30–7.49 (m, 15H, aromatic H), 7.64 (dd, J = 2.0, 8.6 Hz, 1H, 6′-H), 7.77 (d, J = 2.0 Hz, 1H, 2′-H). ESI-MS m/z: 601 $[M + H]^+$, 623 $[M + Na]^+$.

3.1.26. 2-(3,4-Dihydroxyphenyl)-7-hydroxy-3,5-dimethoxy-4H-chromen-4-one (**23**)

To a solution of **29** (100 mg, 0.17 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 12 h, the reaction mixture was filtered through celite and the filtrate concentrated. The crude material was then chromatographed over silica gel (50% ethyl acetate in petroleum ether) to yield **23** (51 mg, 91%) as a yellow solid. Mp 263–264 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.70 (s, 3H, $-OCH_3$), 3.80 (s, 3H, $-OCH_3$), 6.34 (d, J = 2.0 Hz, 1H, 6-H), 6.43 (d, J = 2.0 Hz, 1H, 8-H), 6.87 (d, J = 8.4 Hz, 1H, 5′-H), 7.37 (dd, J = 2.2, 8.4 Hz, 1H, 6′-H), 7.49 (d, J = 2.2 Hz, 1H, 2′-H), 9.28 (s, 1H, 3′-OH), 9.56 (s, 1H, 4′-OH), 10.67 (s, 1H, 7-OH). ESI-MS m/z: 331 $[M + H]^+$, 353 $[M + Na]^+$.

3.1.27. 3-(Benzyloxy)-2-(2,2-diphenylbenzo[d][1,3]dioxol-5-yl)-5,7-dimethoxy-4H-chromen-4-one (**30**)

To a solution of **17** (278 mg, 0.5 mmol) in dry DMF (20 ml) was added K_2CO_3 (414 mg, 3.0 mmol, 6.0 equiv.) and iodomethane (0.16 ml, 2.5 mmol, 5.0 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, dried over $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl

acetate in petroleum ether) to yield **30** (269 mg, 92% yield) as a yellow solid. Mp 195–196 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.91 (s, 3H, $-OCH_3$), 3.99 (s, 3H, $-OCH_3$), 5.07 (s, 2H, $-OCH_2Ph$), 6.37 (d, J = 2.2 Hz, 1H, 6-H), 6.49 (d, J = 2.2 Hz, 1H, 8-H), 6.92 (d, J = 8.4 Hz, 1H, 5′-H), 7.41–7.45 (m, 6H, aromatic H), 7.56 (dd, J = 1.7, 8.4 Hz, 1H, 6′-H), 7.60 (d, J = 1.7 Hz, 1H, 2′-H). 7.61–7.64 (m, 4H, aromatic H). ESI-MS m/z: 585 $[M + H]^+$, 607 $[M + Na]^+$.

3.1.28. 2-(3,4-Dihydroxyphenyl)-3-hydroxy-5,7-dimethoxy-4H-chromen-4-one (**24**)

To a solution of **30** (100 mg, 0.17 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 12 h, the reaction mixture was filtered through celite and the filtrate concentrated. The crude material was then chromatographed over silica gel (50% ethyl acetate in petroleum ether) to yield **24** (52 mg, 92%) as a yellow solid. Mp >300 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.86 (s, 3H, $-OCH_3$), 3.89 (s, 3H, $-OCH_3$), 6.47 (d, J = 2.0 Hz, 1H, 6-H), 6.76 (d, J = 2.0 Hz, 1H, 8-H), 6.88 (d, J = 8.4 Hz, 1H, 5′-H), 7.52 (dd, J = 2.0, 8.4 Hz, 1H, 6′-H), 7.68 (d, J = 2.0 Hz, 1H, 2′-H). ESI-MS m/z: 331 $[M + H]^+$, 353 $[M + Na]^+$.

3.1.29. Synthesis of dimethylether **25** and trimethylether **31**

To a solution of **1** (151 mg, 0.5 mmol) in dry DMF (20 ml) was added K_2CO_3 (276 mg, 2.0 mmol, 4.0 equiv.) and iodomethane (0.11 ml, 1.75 mmol, 3.5 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 $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield **25** (46 mg, 28% yield) and **31** (103 mg, 60% yield) as yellow solids.

3.1.30. 3,5-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-methoxy-4H-chromen-4-one (**25**)

Mp 185–186 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.86 (s, 3H, $-OCH_3$), 3.98 (s, 3H, $-OCH_3$), 5.70 (s, 1H, 3′-OH), 6.28 (d, J = 2.0 Hz, 1H, 6-H), 6.41 (d, J = 2.0 Hz, 1H, 8-H), 6.98 (d, J = 8.6 Hz, 1H, 5′-H), 7.68 (d, J = 2.0 Hz, 1H, 2′-H), 7.78 (dd, J = 2.0, 8.6 Hz, 1H, 6′-H), 12.68 (s, 1H, 5-OH). ESI-MS m/z: 331 $[M + H]^+$, 353 $[M + Na]^+$.

3.1.31. 5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one (**31**)

Mp 148–149 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.87 (s, 6H, $-OCH_3$), 3.99 (s, 3H, $-OCH_3$), 5.70 (s, 1H, $-OH$), 6.35 (d, J = 2.2 Hz, 1H, 6-H), 6.44 (d, J = 2.2 Hz, 1H, 8-H), 6.96 (d, J = 8.6 Hz, 1H, 5′-H), 7.69 (d, J = 2.0 Hz, 1H, 2′-H), 7.78 (dd, J = 2.0, 8.6 Hz, 1H, 6′-H), 12.63 (s, 1H, 5-OH). ESI-MS m/z: 345 $[M + H]^+$, 367 $[M + Na]^+$.

3.1.32. 2-(3,4-Dimethoxyphenyl)-5-methoxy-3,7-bis(methoxymethoxy)-4H-chromen-4-one (**35**)

To a solution of **11** (195 mg, 0.5 mmol) in dry DMF (20 ml) was added K_2CO_3 (497 mg, 3.6 mmol, 7.2 equiv.) and iodomethane (0.20 ml, 3.0 mmol, 6.0 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 $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield **35** (194 mg, 90% yield) as a yellow solid. Mp 148–150 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.23 (s, 3H, $-OCH_3$), 3.52 (s, 3H, $-OCH_3$), 3.96 (s, 3H, $-OCH_3$), 3.97 (s, 3H, $-OCH_3$), 3.98 (s, 3H, $-OCH_3$), 5.21 (s, 2H, $-OCH_2O-$), 5.27 (s, 2H, $-OCH_2O-$), 6.44 (d, J = 2.2 Hz, 1H, 6-H), 6.73 (d, J = 2.2 Hz, 1H, 8-H), 6.97 (d, J = 8.6 Hz, 1H, 5′-H), 7.67–7.69 (m, 2H). ESI-MS m/z: 433 $[M + H]^+$, 455 $[M + Na]^+$.

3.1.33. 2-(3,4-Dimethoxyphenyl)-3,7-dihydroxy-5-methoxy-4H-chromen-4-one (**32**)

To a stirring solution **35** (432 mg, 1 mmol) of in CH_2Cl_2 (5 ml) and ethyl ether (5 ml) was added hydrochloric acid (1 ml) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred another 6 h, then the reaction mixture was diluted with a large amount of ethyl acetate and washed with water and brine. The ethyl acetate layer was dried over $MgSO_4$, filtered and concentrated and the crude material was purified by column chromatography (50% ethyl acetate in petroleum ether) to yield **32** (316 mg, 92%) as a yellow solid. Mp 287–288 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.83 (s, 9H, 3× $-OCH_3$), 6.37 (d, J = 2.0 Hz, 1H, 6-H), 6.55 (d, J = 2.0 Hz, 1H, 8-H), 7.12 (d, J = 8.6 Hz, 1H, 5′-H), 7.73 (d, J = 2.0 Hz, 1H, 2′-H), 7.75 (dd, J = 2.0, 8.6 Hz, 1H, 6′-H), 8.80 (s, 1H, 3-OH), 10.71 (s, 1H, 7-OH). ESI-MS m/z: 345 $[M + H]^+$, 367 $[M + Na]^+$.

3.1.34. 2-(3,4-Dimethoxyphenyl)-3,5-dihydroxy-7-methoxy-4H-chromen-4-one (**33**)

To a stirring mixture of rutin (**13**) (0.626 g, 1 mmol) which has been taken off the three crystal water and K_2CO_3 (0.58 g, 4.2 mmol, 4.2 equiv.) in DMF (30 ml) at room temperature was added iodomethane (0.23 ml, 3.5 mmol, 3.5 equiv.), then the reaction mixture was allowed to warm to 40 °C and the stirring was maintained for 3 h under argon. The resulting mixture was cooled to 0 °C and its pH was adjusted to 6 with 10% acetic acid, then the solid obtained was filtered, dissolved in 20 ml of 95% ethanol and reacted with 3 ml of 36% hydrochloric acid at 70 °C for 2 h. The reaction mixture was cooled to 0 °C, the solid obtained was filtered, washed with cold water and purified by column chromatography (25% ethyl acetate in petroleum ether) to give **33** (0.313 g, 91% yield) as a yellow solid. Mp 176–177 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.89 (s, 3H, $-OCH_3$), 3.97 (s, 3H, $-OCH_3$), 3.99 (s, 3H, $-OCH_3$), 6.38 (d, J = 2.2 Hz, 1H, 6-H), 6.50 (d, J = 2.2 Hz, 1H, 8-H), 7.00 (d, J = 8.6 Hz, 1H, 5′-H), 7.78 (d, J = 2.0 Hz, 1H, 2′-H), 7.83 (dd, J = 2.0, 8.6 Hz, 1H, 6′-H), 11.71 (s, 1H, 5-OH). ESI-MS m/z: 345 $[M + H]^+$, 367 $[M + Na]^+$.

3.1.35. Synthesis of trimethylether **31**, tetramethylether **34** and pentamethylether **37**

To a solution of **1** (151 mg, 0.5 mmol) in dry DMF (20 ml) was added K_2CO_3 (414 mg, 3.0 mmol, 6.0 equiv.) and iodomethane (0.17 ml, 2.75 mmol, 5.5 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 $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (25% ethyl acetate in petroleum ether) to yield trimethylether **31** (34 mg, 20% yield) tetramethylether **34** (57 mg, 32% yield) and pentamethylether **37** (76 mg, 41% yield) as yellow solids.

3.1.36. 2-(3,4-Dimethoxyphenyl)-5-hydroxy-3,7-dimethoxy-4H-chromen-4-one (**34**)

Mp 156–157 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.87 (s, 3H, $-OCH_3$), 3.88 (s, 3H, $-OCH_3$), 3.97 (s, 3H, $-OCH_3$), 3.98 (s, 3H, $-OCH_3$), 6.36 (d, J = 2.2 Hz, 1H, 6-H), 6.45 (d, J = 2.2 Hz, 1H, 8-H), 6.99 (d, J = 8.6 Hz, 1H, 5′-H), 7.69 (d, J = 2.0 Hz, 1H, 2′-H), 7.74 (dd, J = 2.0, 8.6 Hz, 1H, 6′-H), 12.64 (s, 1H, 5-OH). ESI-MS m/z: 359 $[M + H]^+$, 381 $[M + Na]^+$.

3.1.37. 2-(3,4-Dimethoxyphenyl)-3,5,7-trimethoxy-4H-chromen-4-one (**37**)

Mp 136–137 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.85 (s, 3H, $-OCH_3$), 3.90 (s, 3H, $-OCH_3$), 3.97 (s, 9H, 3×$-OCH_3$), 6.35 (d, J = 2.2 Hz, 1H, 6-H), 6.51 (d, J = 2.2 Hz, 1H, 8-H), 6.98 (d, J = 9.2 Hz, 1H, 5′-H), 7.69 (dd, J = 2.0, 9.2 Hz, 1H, 6′-H), 7.72 (d, J = 2.0 Hz, 1H, 2′-H). ESI-MS m/z: 373 $[M + H]^+$, 395 $[M + Na]^+$.

3.2. Anti-thrombic assay

3.2.1. 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.

3.2.2. Plasma anticoagulation assay

Thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen content (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. Assaying the prolongation of the plasma clotting time of TT, APTT, INR increasement of PT, and reduction of FIB content assessed the anticoagulation activity.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (30873235, 81001382), Program for New Century Excellent Talents by the Ministry of Education (NCET-09-0163), Research Fund for the Doctoral Program of Higher Education of China (20093237120012), the Main Training Fund of Nanjing University of Chinese Medicine (10XPY02), 333 High-level Talents Training Project Funded by Jiangsu Province, Six Talents Project Funded by Jiangsu Province (2011-D-078), Program for Outstanding Scientific and Technological Innovation Team of Jiangsu Higher Education (2009), Key Research Project in Basic Science of Jiangsu College and University (NO. 06KJA36022, 07KJA36024, 10KJA360039), Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (ysxk-2010), and Construction Project for Jiangsu Engineering Center of Innovative Drug from Blood-conditioning TCM Formulae.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2012.04.044. These data include MOL files and InChIKeys of the most important compounds described in this article.

References

[1] J.I. Weitz, M. Crowther, Thromb. Res. 106 (2002) V275–V284.
[2] J.I. Weitz, Thromb. Res. 109 (2003) S17–S22.
[3] S. Hanessian, D. Simard, M. Bayrakdarian, E. Therrien, I. Nilsson, O. Fjellström, Bioorg. Med. Chem. Lett. 18 (2008) 1972–1976.
[4] S.V. Manoukian, F. Feit, R. Mehran, M.D. Voeltz, R. Ebrahimi, M. Hamon, G.D. Dangas, A.M. Lincoff, H.D. White, J.W. Moses, S.B. King III, E.M. Ohman, G.W. Stone, J. Am. Coll. Cardiol. 49 (2007) 1362–1368.
[5] H.X. Li, S.Y. Han, X.W. Wang, X. Ma, K. Zhang, L. Wang, Z.Z. Ma, P.F. Tu, Food Chem. Toxicol. 47 (2009) 1797–1802.
[6] Y. Liu, X.Y. Lai, X.M. Ling, Y.Y. Zhao, J.R. Cui, Chromatographia 64 (2006) 45–50.
[7] B. Singh, P. Kaur, Gopichand, R.D. Singh, P.S. Ahuja, Fitoterapia 79 (2008) 401–418.
[8] L. Liu, H.Y. Ma, N.Y. Yang, Y.P. Tang, J.M. Guo, W.W. Tao, J.A. Duan, Thromb. Res. 126 (2010) e365–e378.
[9] W. Bors, C. Michel, K. Stettmaier, in: L. Packer (Ed.), Meth. Enzymol., 335, Academic Press, San Diego, 2001, pp. 166–180.
[10] P. Ader, A. Wessmann, S. Wolffram, Free Rad. Biol. Med. 28 (2000) 1056–1067.
[11] C. Manach, O. Texier, C. Morand, V. Crespy, F. Régérat, C. Demigné, C. Rémésy, Free Rad. Biol. Med. 27 (1999) 1259–1266.
[12] D.W. Boulton, U.K. Walle, T. Walle, J. Pharm. Pharmacol. 51 (1999) 353–359.
[13] K.A. O'Leary, A.J. Day, P.W. Needs, W.S. Sly, N.M. O'Brien, G. Williamson, FEBS Lett. 503 (2001) 103–106.
[14] T. Koga, M. Meydani, Am. J. Clin. Nutr. 73 (2001) 941–948.
[15] N.G. Li, Z.H. Shi, Y.P. Tang, J.P. Yang, J.A. Duan, Beilstein J. Org. Chem. 5 (2009) 1–5.
[16] M. Bouktaib, S. Lebrun, A. Atmani, C. Rolando, Tetrahedron 58 (2002) 10001–10009.
[17] N.G. Li, J.X. Wang, X.R. Liu, C.J. Lin, Q.D. You, Q.L. Guo, Tetrahedron Lett. 48 (2007) 6586–6589.
[18] H.J. Li, X.H. Luan, Y.M. Zhao, Chin. J. Org. Chem. 24 (2004) 1619–1621.
[19] R.C. Isaacs, M.G. Solinsky, K.J. Cutrona, C.L. Newton, A.M. Naylor-Olsen, J.A. Krueger, S.D. Lewis, B.J. Lucas, Bioorg. Med. Chem. Lett. 16 (2006) 338–342.
[20] R.C. Isaacs, M.G. Solinsky, K.J. Cutrona, C.L. Newton, A.M. Naylor-Olsen, D.R. McMasters, J.A. Krueger, S.D. Lewis, B.J. Lucas, L.C. Kuo, Y. Yan, J.J. Lynch, E.A. Lyle, Bioorg. Med. Chem. Lett. 18 (2008) 2062–2066.