Synthesis of 3,15-Disuccinate-12-Coumarin Substituted Andrographolide Derivatives and Their Antiplatelet Aggregation Activities In Vitro

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Abstract
In order to develop a series of novel compounds with antiplatelet aggregation activities, 3,15-disuccinate-12-coumarin substituted derivatives were designed and synthesized based on the natural product andrographolide. In vitro antiplatelet aggregation activities were evaluated by the turbidimetric method with thrombin, adenosine diphosphate (ADP), arachidonic acid (AA), and collagen as inducers. The biological evaluation revealed that compound 11k showed significant inhibition activity for thrombin, AA, and collagen-induced platelet aggregation at the same time and exhibited a dose-dependent behavior. Compound 11c showed the highest antiplatelet aggregation activity induced by ADP. Most of the derivatives had no significant cytotoxicity. Therefore, our work proved that 3,15-disuccinate-12-coumarin substituted andrographolide derivatives had the potential to become a novel candidate structure for antiplatelet aggregation and deserved further study.

Keywords
andrographolide, Design, 3,15-Disuccinate-12-coumarin substituted andrographolide derivativesx, synthesis, anti-platelet aggregation

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Thrombus is a quite common cardiovascular disease and seriously threatens human health and life quality.1–3 Platelet aggregation is one of the important factors leading to thrombosis. Antiaggregation drugs are often used as the top-priority drugs to treat thrombosis.4,5 Currently, a variety of antiplatelet aggregation drugs have been successfully marketed. According to different mechanisms, there are thromboxane A2 inhibitor (aspirin),5 phosphodiesterase inhibitor (cilostazol),6 5-hydroxytryptamine receptor antagonist (sarpogrelate),7 platelet membrane glycoproteins IIb/IIIa receptor inhibitor (tirofiban),8 adenosine diphosphate (ADP) induction inhibitor (clopidogrel),9 Ca2+ channel antagonist (flunarizine),10–12 and protease active receptor-1 (PAR-1) agonist (vorapaxar sulfate).13

Andrographolide 1 (Figure 1), a labdane diterpene, is abundant in the aerial parts of Andrographis paniculata Nees with up to 2.0%.14 Andrographolide possesses rich biological activities such as antiproliferation,15 antitumorotropic,16 antimalaria,17 anti-HIV,18 anticancer,19 antibacterial,20 antithrombosis,21 anti-inflammatory,22 anti-influenza virus,23 and antihyperglycemia.24 In recent years, there were a few publications reporting A. paniculata extract and its active diterpenoids possessing antiplatelet aggregation activity.25–27 In 2011 and 2013, Lien et al and Lu et al illustrated possible mechanisms of antiplatelet aggregation of andrographolide such as regulating the extrinsic apoptotic or inhibiting endothelial nitric oxide synthase/cyclic guanosine monophosphate.28,29 In 2017, Liu et al developed a series of novel and potent orally active PAR1 derivatives based on andrographolide and vorapaxar.30 In our previous work, we had found that andrographolide and dehydroandrographolide 2 (Figure 1) had low antiplatelet aggregation activities with ADP as inducer. Their sulfonate 3 (medicinal composition of Xiyanping) and succinate 4 (Figure 1) (medicinal composition of Chuanhuning) showed better antiplatelet aggregation activities, especially the latter,31,32 the reason for which were that andrographolide and dehydroandrographolide possessed poor
aqueous solubility, but their sulfonate and succinate possessed high aqueous solubility.34,35

The well-known Coumarins, easily available natural compounds, exhibit a wide range of biological activities such as acetylcholinesterase inhibitor,36 antioxidant,37 antitumor,38 anti-inflammatory,39 alpha-chymotrypsin inhibitor,40 and anti-HIV.41 In addition, coumarin derivatives have obvious anti-platelet aggregation activities.42-46 Among them, warfarin 5, dicoumarol 6, and acenocoumarol 7 (Figure 1) are marketed drugs for treating thromboembolic diseases.47,48

Based on the above, it is highly worthwhile to develop accessible and effective methods to construct novel antiplatelet aggregation compounds with andrographolide and coumarins.

Results and Discussion

Preparation of Target Compounds 11

By analyzing the structure, andrographolide had many reaction sites such as 3,19-OH, C-8 = C-17, C-12, C-15. Based on our previous work, to improved aqueous solubility, 2 molecules of carboxylates were still introduced at 3,19-OH. In addition, coumarin derivatives have obvious anti-platelet aggregation activities.42-46 Among them, warfarin 5, dicoumarol 6, and acenocoumarol 7 (Figure 1) are marketed drugs for treating thromboembolic diseases.47,48

Based on the above, it is highly worthwhile to develop accessible and effective methods to construct novel antiplatelet aggregation compounds with andrographolide and coumarins.

Figure 1. Anti-platelet aggregation compounds (Andrographolides and Coumarins).
introduced at 3,19-OH to give the intermediate 8. Afterward, intermediate 8 degraded to the key intermediate 9 by selective oxidation of the C-12,13 olefin bond under potassium permanganate (KMnO₄). Lastly, target derivatives (3,15-disuccinate-12-coumarin substituted andrographolide derivatives) were synthesized from intermediate 9 reacting with 4-methylcoumarins under potassium tert-butoxide (t-BuOK).

Biological Properties

In vitro antiplatelet aggregation activities of 11a-11l were assessed using Sprague Dawley (SD) male rats arterial blood by the turbidimetric method. Thrombin, ADP, arachidonic acid (AA), and collagen were used as inducers. Aspirin was employed to be the positive control. All the results were summarized in Table 1. Those compounds with significant antiplatelet aggregation activities were chosen to investigate the relationship between activity and concentration (Figure 2).

Compared Entry 3 with Entry 4, the IR of compound 4 was obviously higher than compound 3, which revealed that succinic acid salinization could be more beneficial to improve the activities of derivatives. Therefore, in our current work, all the derivatives were succinated at 3,19-OH (Scheme 1).

For thrombin, AA, and collagen as inducers, 11c, 11g, 11i, 11k, and 11l showed prominent antiplatelet aggregation activities equivalent or superior to the positive control. The same substituents attaching to different positions would lead to different activities. Analyzing Entries 6, 9, and 13, the order of activity was 7′-CH₃ (11i) > 6′-CH₃ (11e) > 3′-CH₃ (11b). Analyzing Entries 8, 12, and 16, the order of activity was 7′-Cl (11l) > 6′-Cl (11h) > 3′-Cl (11d). Over all, the activities of 7′-substituted derivatives (11i-11l) were higher than those of 3′-substituted (11b-11d) and 6′-substituted (11e-11h) derivatives. Different substituents attaching to the same positions also resulted in different activities. When attaching to 3′ position, the order of inhibition rate was 3′-COOCH₂CH₃ (11c) > 3′-CH₃ (11b) > 3′-Cl (11d) (for thrombin, AA, and collagen). When attaching to 6′ position, the orders of inhibition rate were 6′-OCOCH₃ (11g) > 6′-CH₃ (11e) > 6′-Cl (11h) > 6′-OCH₃ (11f) (for thrombin), 7′-OCOCH₃ (11g) > 6′-Cl (11h) > 6′-CH₃ (11e) > 6′-OCH₃ (11f) (for AA and collagen). When attaching to 7′ position, the orders of inhibition rate were 7′-OCOCH₃ (11k) > 7′-CH₃ (11i) > 7′-Cl (11l) > 7′-OCH₃ (11j) (for thrombin), 7′-OCOCH₃ (11k) > 7′-Cl (11l) > 7′-CH₃ (11i) > 7′-OCH₃ (11j) (for AA), and 7′-OCOCH₃ (11k) > 7′-OCH₃ (11j) > 7′-Cl (11l) > 7′-CH₃ (11i) (for collagen). The electron effect of substituents may had little impact on activities. When methoxyl and chloro groups were introduced into 3′, 6′, 7′ positions, antiplatelet aggregation activities would subside, especially for thrombin and AA. Interestingly, 11k strongly inhibited thrombin, AA, and collagen-induced platelet aggregation at the same time and exhibited a dose-dependent behavior (Figure 2(a, c, d)).

For ADP as inducer, only 11c displayed good antiplatelet aggregation activities and also exhibited a dose-dependent behavior (Figure 2(b)). The activities of 3′-substituted derivatives (11b-11d) were higher than the 6′-substituted (11e-11h) and 7′-substituted (11i-11l). When chloro group attaching to 3′, 6′, 7′ positions, antiplatelet aggregation activities also subsided.

Scheme 1. The synthetic route of derivatives 11. Reagents and conditions: (a) pyridine, succinic anhydride, reflux, 12 hours, then, 4 M hydrochloric acid, 60°C, 30 minutes; (b) potassium permanganate, tetrahydrofuran, −5°C; (c) potassium tert-butoxide, 4-methylcoumarins, dimethyl sulfoxide, room temperature, 5 hours, then, acetic acid, 1 hour, then potassium bicarbonate.
Table 1. The IR and IC\\(_{50}\\) of Target Compounds 11 In Vitro.

| Entry | Compounds | Dose (μM) | Thrombin (0.1 U/mL) | ADP (5 mM) | AA (20 μM) | Collagen (1 mg/mL) |
|-------|-----------|-----------|---------------------|------------|------------|-------------------|
|       |           | IR (%)\(^a\) | IC\\(_{50}\\) (μM)\(^b\) | IR (%)\(^a\) | IC\\(_{50}\\) (μM)\(^b\) | IR (%)\(^a\) | IC\\(_{50}\\) (μM)\(^b\) |
| 1     | Aspirin   | 1.7       | 24.32 ± 0.25        | -          | 54.03 ± 0.15 | 0.27 | 38.33 ± 0.13 | 0.44 | 49.27 ± 0.17 | 0.47 |
| 2     | 1         | 1.7       | 23.55 ± 0.23**      | -          | 26.64 ± 0.14* | -   | 21.46 ± 0.10 | -   | 31.48 ± 0.15* | -   |
| 3     | 3         | 1.7       | 30.31 ± 0.16*       | -          | 38.25 ± 0.16** | -   | 27.65 ± 0.12 | -   | 36.63 ± 0.12 | -   |
| 4     | 4         | 1.7       | 35.56 ± 0.14**      | -          | 42.54 ± 0.14  | 0.71 | 30.48 ± 0.15* | -   | 41.78 ± 0.13 | -   |
| 5     | 11a       | 1.7       | 38.71 ± 0.16**      | -          | 41.65 ± 0.12  | 0.86 | 31.58 ± 0.16 | -   | 38.56 ± 0.10* | -   |
| 6     | 11b       | 1.7       | 42.57 ± 0.22        | 0.72       | 47.71 ± 0.11  | 0.65 | 23.56 ± 0.12 | -   | 37.55 ± 0.14 | -   |
| 7     | 11c       | 1.7       | 46.87 ± 0.14*       | 0.53       | 53.69 ± 0.15** | 0.31 | 35.75 ± 0.17 | 0.72 | 39.36 ± 0.17* | -   |
| 8     | 11d       | 1.7       | 36.67 ± 0.21*       | -          | 42.18 ± 0.11** | 0.68 | 20.86 ± 0.10 | -   | 33.78 ± 0.10 | -   |
| 9     | 11e       | 1.7       | 44.83 ± 0.09**      | 0.59       | 37.25 ± 0.18** | -   | 31.47 ± 0.13 | -   | 44.57 ± 0.16 | 0.81 |
| 10    | 11f       | 1.7       | 41.14 ± 0.05*       | 0.67       | 44.67 ± 0.06* | 0.75 | 30.15 ± 0.12 | -   | 42.75 ± 0.14* | -   |
| 11    | 11g       | 1.7       | 47.25 ± 0.06        | 0.53       | 43.13 ± 0.18  | 0.46 | 37.25 ± 0.13* | 0.68 | 49.86 ± 0.18 | 0.70 |
| 12    | 11h       | 1.7       | 42.36 ± 0.13**      | 0.42       | 38.75 ± 0.09** | 0.64 | 35.29 ± 0.13 | 0.63 | 47.57 ± 0.15 | 0.67 |
| 13    | 11i       | 1.7       | 47.74 ± 0.21*       | 0.51       | 43.47 ± 0.14  | 0.73 | 36.66 ± 0.13 | 0.57 | 48.76 ± 0.14 | 0.59 |
| 14    | 11j       | 1.7       | 43.38 ± 0.18        | 0.46       | 40.54 ± 0.21** | 0.81 | 33.43 ± 0.16 | -   | 51.72 ± 0.10 | 0.54 |
| 15    | 11k       | 1.7       | 50.14 ± 0.17**      | 0.36       | 41.69 ± 0.13  | 0.68 | 46.73 ± 0.12* | 0.48 | 56.24 ± 0.13* | 0.50 |
| 16    | 11l       | 1.7       | 45.49 ± 0.07*       | 0.68       | 36.05 ± 0.22* | -   | 42.48 ± 0.13 | 0.56 | 50.55 ± 0.14* | 0.69 |

ADP, adenosine diphosphate; AA, arachidonic acid; IC\\(_{50}\\), half-maximal inhibitory concentration.

\(^*P<0.05\) vs aspirin. \(^**P<0.01\) vs aspirin.

\(^a\) IRs were expressed as the means ± standard error of 6 experimental replicates (\(n=6\)).

\(^b\) "–", not tested.
To sum up, for thrombin, AA, and collagen as inducers, 11k (for thrombin, IR: 50.14%, half-maximal inhibitory concentration [IC50]: 0.36 µM; for AA, IR: 46.73%, IC50: 0.48 µM; for collagen, IR: 56.24%, IC50: 0.50 µM) showed the best antiplatelet aggregation activity. For ADP as inducer, 11c (IR: 53.69%, IC50: 0.31 µM) displayed the highest antiplatelet aggregation activity and was nearly equivalent to the positive control drug aspirin.

Figure 2. Antiplatelet aggregation activities of compounds 11 at different concentrates (final concentration: 1.7, 3.4, and 6.8 µM). (a) Thrombin, (b) adenosine diphosphate (ADP), (c) arachidonic acid (AA), and (d) collagen. Data were expressed as the means ± standard error of 6 experimental replicates (n = 6).

Cytotoxicity Assay In Vitro

Mouse fibroblast cells (L929) were selected to evaluate cell toxicity of target derivatives 11. The absorbance and survival rates were summarized in Table 2. The result revealed that most of derivatives had no significant cytotoxicity. Among them, the survival rates of 11c, 11e, 11j, and 11k were higher than aspirin at the dose of 10 and 100 µM.
Conclusion

Through our efforts, 3,15-disuccinate-12-coumarin substituted derivatives 11 were designed and synthesized based on the natural product andrographolide. By preliminary biological evaluation, these synthesized compounds showed prominent antiplatelet aggregation activities in response to thrombin, ADP, AA, and collagen agonists in vitro. Compound 11k showed the best activities when thrombin, AA, and collagen were inducers. Among them, compound 11c had the highest antiplatelet aggregation activities when ADP was inducer. Most of the derivatives had no significant cytotoxicity. Therefore, our research results proved that 3,15-disuccinate-12-coumarin substituted andrographolide derivatives had the potential to become a novel candidate structure for antiplatelet aggregation. The most active compounds were to be selected to measure bleeding time and mechanisms of action in later work.

Experimental

General

Infrared spectra were determined on a Nicolet Avatar-370 spectrometer in potassium bromide (ν in cm⁻¹). Melting points were measured on a Büchi B-540 capillary melting point apparatus and uncorrected. Electrospray ionization-mass spectra (ESI-MS) were determined on a Thermo Finnigan LCQ- Advantage. High-resolution MS (HRMS) was determined on an Agilent 6210 TOF instrument. 1H nuclear magnetic resonance (NMR) and 13C NMR spectra were recorded on Varian Mercury Plus-400 spectrometer (400 and 100 MHz) in dimethyl sulfoxide (DMSO)-d₆ or D₂O, δ in parts per million, J in Hertz, using tetramethylsilane as an internal standard. Platelet aggregation rates were measured on LG-PABER Platelet aggregation apparatus (made in Beijing Shidi Scientific Instrument Co. LTD, Beijing). Column chromatography was carried out on silicagel (200-300 grading). Andrographolide 1 was provided by Chengdu Biochemical Biotechnology Co. LTD (Chengdu, China). All other analytical reagents like pyridine, tetrahydrofuran (THF), methanol (MeOH), acetic acid (AcOH), DMSO, succinic anhydride, KMnO₄, t-BuOK, 4-methylcoumarins were commercially available and used directly without further purification.

Synthesis of Derivatives

Synthesis of intermediate 8. Andrographolide 1 (60.0 g, 171.3 mmol) was dissolved in anhydrous pyridine (200 mL) and then succinic anhydride (48.0 g, 479.6 mmol) was added. The mixture was stirred at reflux until total consumption of the starting material about 12 hours under monitoring of thin-layer chromatography (TLC). Afterward, most of the pyridine should be evaporated under reduced pressure. The mixture cooled to 60°C and was poured to 4M hydrochloric acid slowly with stirring vigorously for 30 minutes to keep pH = 5-6. Lastly, the mixture was filtered and washed with water. The residue was provided by Chengdu Biochemical Biotechnology co. LTD (Chengdu, China). Column chromatography was carried out on silicagel (200-300 grading). Andrographolide 1 was provided by Chengdu Biochemical Biotechnology Co. LTD (Chengdu, China). All other analytical reagents like pyridine, tetrahydrofuran (THF), methanol (MeOH), acetic acid (AcOH), DMSO, succinic anhydride, KMnO₄, t-BuOK, 4-methylcoumarins were commercially available and used directly without further purification.

Table 2. The Absorbance and Survival Rates of Target Compounds 11 In Vitro.

| Compounds | Dose (μM) | Absorbance | Survival rate (%)a | Compounds | Dose (μM) | Absorbance | Survival rate (%)a |
|-----------|-----------|------------|-------------------|-----------|-----------|------------|-------------------|
| Blank group | -         | 0.202      | -                 | 1f        | 10        | 0.240      | 68.30 ± 0.06*     |
|           | 100       | 0.229      |                  |           | 100       | 0.251      | 51.64 ± 0.06*     |
| Control group | -         | 0.258      | -                 | 1g        | 10        | 0.241      | 70.35 ± 0.06*     |
|           | 100       | 0.229      |                  |           | 100       | 0.231      | 47.67 ± 0.07      |
| Aspirin   | 10        | 0.242      | 72.02 ± 0.08     | 1h        | 10        | 0.244      | 74.08 ± 0.08      |
|           | 100       | 0.232      | 54.33 ± 0.06*    |           | 100       | 0.229      | 47.67 ± 0.06      |
| 1a        | 10        | 0.239      | 66.02 ± 0.06     | 1i        | 10        | 0.245      | 77.23 ± 0.10*     |
|           | 100       | 0.226      | 43.56 ± 0.09*    |           | 100       | 0.232      | 54.33 ± 0.06      |
| 1b        | 10        | 0.238      | 64.60 ± 0.11     | 1j        | 10        | 0.247      | 79.69 ± 0.09*     |
|           | 100       | 0.226      | 43.56 ± 0.09     |           | 100       | 0.233      | 55.87 ± 0.06      |
| 1c        | 10        | 0.245      | 77.23 ± 0.10*    | 1k        | 10        | 0.245      | 77.23 ± 0.10**    |
|           | 100       | 0.237      | 62.31 ± 0.05     |           | 100       | 0.236      | 60.30 ± 0.08      |
| 1d        | 10        | 0.245      | 77.23 ± 0.10*    | 1l        | 10        | 0.241      | 70.35 ± 0.06*     |
|           | 100       | 0.231      | 51.64 ± 0.06     |           | 100       | 0.227      | 45.37 ± 0.08      |
| 1e        | 10        | 0.247      | 79.69 ± 0.09**   |           | 100       | 0.238      | 64.60 ± 0.11      |
|           |           |            |                  |           |           |            |                   |

aP < 0.05 vs aspirin. **P < 0.01 vs aspirin.

Survival rates were expressed as the means ± standard error of 6 experimental replicates (n = 6).
for about 2 hours. After reaction, sodium bicarbonate solution and water (15 mL, 3:1) was added to the mixture with stirring under the monitoring of TLC. Then a mixed solution of AcOH (8 mmol) was added. The mixture was stirred at room temperature in dry DMSO (20 mL) and then 4-methylcoumarin (1.1 g, 6.6 mmol) and t-BuOK (0.64 g, 5.7 mmol) were dissolved (2.0 g, 4.4 mmol) and 1,2,5,6,7H), 1.05 (3H, s, 14H), 0.97 (3H, s, 16H). 13C NMR (100 MHz, D2O): \( \delta = 174.7 \) (C-20), 174.6 (C-24), 173.2 (C-17), 173.1 (C-21), 162.2 (C-4), 159.3 (C-2′), 150.1 (C-9), 148.8 (C-8), 135.5 (C-11), 129.1 (C-5′), 128.3 (C-7′), 128.0 (C-12), 127.2 (C-10′), 125.4 (C-6′), 111.2 (C-8′), 109.1 (C-13), 104.7 (C-3′), 75.0 (C-3′), 65.9 (C-15), 60.9 (C-9′), 47.5 (C-5′), 40.9 (C-4′), 39.8 (C-10′), 36.7 (C-3′), 33.6 (C-1), 29.5 (C-18), 29.2 (C-22), 29.1 (C-19), 29.0 (C-23), 24.3 (C-2′), 24.0 (C-6′), 21.9 (C-14), 14.7 (C-16) ppm. MS (ESI): \( m/z (\%) = 533.2 ([M + H]^+) \), 100%. HRMS (ESI) calced for C28H37O10 [M + H]^+ : 533.2388; found 533.2396.

**Synthesis of intermediate 9.** Intermediate 8 (48.0 g, 90.2 mmol) was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added was dissolved in THF (200 mL) and then KMnO4 (21.4 g, 135.3 mmol) was added.
Monopotassium 3,15-disuccinate-12-(6′-methanoylcoumarin) andrographolide 11e. Pale yellow solid, 1.16 g, yield 39.5%, purity 96.4%. 1H NMR (400 MHz, D2O): δ = 7.04-7.01 (1H, m, 5′-H), 7.0-7.6 (2H, m, 7′-H, 8′-H). 6.46 (1H, s, 3′-H), 6.43 (1H, d, j = 15.8 Hz, 12H), 5.03 (1H, s, 13H), 4.82 (1H, s, 14H), 4.78 (1H, s, 15H), 4.4 (1H, m, 18H), 3.9 (1H, s, 19H), 3.85-3.78 (3H, m, 4H, 20H, 21H). MS (ESI): m/z (%) = 667.2 ([M + H]+ 100%), 669.2 ([M + H]+ 100%), 671.1712, found 667.1734; for C33H37KO12 [M + H]+: 669.1683, found 669.1698.

Monopotassium 3,15-disuccinate-12-(7′-methylcoumarin) andrographolide 11h. Pale yellow solid, 1.16 g, yield 39.5%, purity 96.4%. 1H NMR (400 MHz, D2O): δ = 7.84-7.81 (1H, m, 5′-H), 7.0-7.6 (2H, m, 7′-H, 8′-H). 6.46 (1H, s, 3′-H), 6.43 (1H, d, j = 15.8 Hz, 12H), 5.03 (1H, s, 13H), 4.82 (1H, s, 14H), 4.78 (1H, s, 15H), 4.4 (1H, m, 18H), 3.9 (1H, s, 19H), 3.85-3.78 (3H, m, 4H, 20H, 21H). MS (ESI): m/z (%) = 667.2 ([M + H]+ 100%), 669.2 ([M + H]+ 100%), 671.1712, found 667.1734; for C33H37KO12 [M + H]+: 669.1683, found 669.1698.

Monopotassium 3,15-disuccinate-12-(6′-methylcoumarin) andrographolide 11e. White solid, 1.14 g, yield 42.0%, purity 95.3%. 1H NMR (400 MHz, D2O): δ = 7.59-7.56 (1H, m, 5′-H), 7.31-7.26 (2H, m, 7′-H, 8′-H). 6.46 (1H, s, 3′-H), 6.19 (1H, d, j = 15.8 Hz, 12H), 5.73 (1H, m, 11H), 5.03 (1H, s, 13H, 4H), 4.86 (1H, s, 13H), 4.14 (1H, d, j = 11.8 Hz, 15-Ha), 3.93-3.83 (2H, m, 3H, 15-Hb, 4′-CH3), 2.83-2.61 (9H, m, 9,18,19,22,23H), 2.34 (3H, s, 14H), 2.24 (3H, s, 14H), 1.6 (9H, m, 1,2,5,6,7H), 1.05 (3H, s, 14H). MS (ESI): m/z (%) = 667.2 ([M + H]+ 100%), 669.2 ([M + H]+ 100%), 671.1712, found 667.1734; for C33H37KO12 [M + H]+: 669.1683, found 669.1698.

Monopotassium 3,15-disuccinate-12-(6′-methylcoumarin) andrographolide 11e. Pale yellow solid, 1.16 g, yield 39.5%, purity 96.4%. 1H NMR (400 MHz, D2O): δ = 7.84-7.81 (1H, m, 5′-H), 7.0-7.6 (2H, m, 7′-H, 8′-H). 6.46 (1H, s, 3′-H), 6.43 (1H, d, j = 15.8 Hz, 12H), 5.03 (1H, s, 13H), 4.82 (1H, s, 14H), 4.78 (1H, s, 15H), 4.4 (1H, m, 18H), 3.9 (1H, s, 19H), 3.85-3.78 (3H, m, 4H, 20H, 21H). MS (ESI): m/z (%) = 667.2 ([M + H]+ 100%), 669.2 ([M + H]+ 100%), 671.1712, found 667.1734; for C33H37KO12 [M + H]+: 669.1683, found 669.1698.

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126.7 (C-5′), 128.0 (C-12), 124.2 (C-10′), 125.7 (C-6′), 117.2 (C-8′), 109.2 (C-13), 104.7 (C-3′), 75.0 (C-3′), 65.9 (C-15), 60.8 (C-9′), 47.5 (C-5′), 40.8 (C-4′), 39.8 (C-10), 36.6 (C-7′), 33.4 (C-1), 29.5 (C-18′), 29.3 (C-22), 29.1 (C-9′), 29.0 (C-23), 24.3 (C-2), 24.0 (C-6′), 21.9 (C-14), 21.2 (7′-CH₃), 14.7 (C-16) ppm. MS (ESI): $m/z$ (%) = 667.2 ([M + H⁺] +, 100%). HRMS (ESI) calcd for $C_{35}H_{40}KO_{12}$ [M + H⁺]: 669.1683, found 669.1601.

**Monopotassium 3,15-disuccinate-12-(7′-chlorocoumarin) androgrobalide 11j.** Pale yellow solid. 1.19 g, yield 39.2%, purity 96.3%. $^1$H NMR (400 MHz, D₂O): $\delta$ = 7.83–7.78 (1H, m, 5′-H), 7.41–7.38 (1H, m, 8′-H), 7.28–7.25 (1H, m, 6′-H), 6.64 (1H, s, 3′-H), 5.03 (1H, s, 7′-H), 4.86 (1H, d, $J = 11.8$ Hz, 15-Ha), 3.93–3.81 (5H, m, 15-Hb, 7′-OCH₃), 2.83–2.52 (9H, m, 9, 18, 19, 22, 23-H), 2.28 (3H, s, 7′-OCOCH₃), 2.11–1.61 (9H, m, 1, 2, 5, 6, 7-H). MS (ESI): $m/z$ (%) = 663.2 ([M + H⁺] +, 100%). HRMS (ESI) calcd for $C_{34}H_{40}KO_{11}$ [M + H⁺]: 663.2208; found 663.2219.

**Monopotassium 3,15-disuccinate-12-(7′-methanoylauronmarin) androgrobalide 11k.** Pale yellow solid. 1.19 g, yield 39.2%, purity 95.2%. $^1$H NMR (400 MHz, D₂O): $\delta$ = 7.83–7.80 (1H, m, 5′-H), 7.41–7.38 (1H, m, 8′-H), 7.28–7.25 (1H, m, 6′-H), 6.64 (1H, s, 3′-H), 6.18 (1H, d, $J = 15.8$ Hz, 12-H), 5.71 (1H, m, 11′-H), 5.03 (1H, s, 13-Ha), 4.86 (1H, d, $J = 11.8$ Hz, 15-Ha), 3.93–3.81 (5H, m, 15-Hb, 7′-OCH₃), 2.83–2.64 (9H, m, 9, 18, 19, 22, 23-H), 2.13–1.67 (9H, m, 1, 2, 5, 6, 7-H), 1.04 (3H, s, 14-H), 0.96 (3H, s, 16-H). $^{13}$C NMR (100 MHz, D₂O): $\delta$ = 174.7 (C-20), 174.6 (C-24), 173.2 (C-17), 173.1 (C-21), 162.1 (C-4′), 160.3 (C-7′), 159.3 (C-2′), 151.1 (C-9′), 148.8 (C-8′), 135.5 (C-11), 130.8 (C-5′), 128.0 (C-12), 119.2 (C-10′), 111.2 (C-6′), 109.1 (C-13), 104.7 (C-3′), 98.2 (C-8′), 75.3 (C-15), 65.9 (C-9′), 55.8 (7′-OCH₃), 47.6 (C-5′), 40.9 (C-4′), 39.8 (C-10), 36.7 (C-7′), 33.4 (C-1), 29.4 (C-18), 29.2 (C-22), 29.1 (C-19), 29.0 (C-23), 24.1 (C-24), 24.0 (C-6′), 21.6 (C-14), 14.7 (C-16) ppm. MS (ESI): $m/z$ (%) = 663.2 ([M + H⁺] +, 100%). HRMS (ESI) calcd for $C_{35}H_{40}KO_{11}$ [M + H⁺]: 663.2208; found 663.2219.

**Biological Assays In Vitro**

Fresh arterial blood was taken from groin of SD male rats with 3.8% sodium citrate as anticoagulant (9:1 by volume). Then, whole blood samples were centrifuged at 1000 rpm/min for 10 minutes at room temperature to give the platelet-rich plasma (PRP). The residue continued to be centrifuged at 3000 rpm/min for 10 minutes to prepare platelet-poor plasma (PPP). PPP was employed to be the blank control. Thrombin, ADP, AA, collagen were employed as inducers. Aspirin was selected to be the positive control. Normal saline was selected as the negative control. The sample groups (5 μL) were prepared with target compounds dissolved in normal saline (1.7 μmol/L, 3.4μmol/L, 6.8μmol/L) and then was added into PRP-2 (200 μL) and incubated for 2 minutes, as well as positive control and negative control. Afterward, adding 20 μL thrombin (0.1 U/mL), ADP (5 mM/L), AA (20μg/mL), collagen (1 mg/mL), respectively, induced platelet aggregation. IR could be calculated by the formula IR = [1 – (SG/NC)] × 100% (SG and NC represented the platelet aggregation rates of sample group and negative control, respectively)

**Cytotoxicity Assay In Vitro**

Cell toxicity of target derivatives 11 were evaluated on mouse fibroblast cells (L929) by Cell Counting Kit-8 (CCK-8) assays.\(^{53}\) First, test compounds were dissolved and diluted into 10 μM/L and 100 μM/L with DMSO, respectively. Cells were added on 96-well microplates (1 × 10⁴ cell/well) and then cultivated at 37°C in a humidified atmosphere of carbon dioxide (5%) for 24 hours. Second, test compound was added into the cells and continued to be incubated at 37°C for 48 hours. Third, the medium was removed and replaced with fresh complete medium of RPMI-1640 (100 μL). CCK-8 solution (10 μL/well) was added to the microplates. Lastly, the absorbance of test solution was measured at 450 nm on Bio-Tek Fluoroskan plate reader. According to the formula to calculate relative survival rate: relative survival rate (%) = \([\text{Abs(test solution)} - \text{Abs(blank cells)}] / [\text{Abs(controlled cells)} - \text{Abs(blank cells)}]\) × 100%.

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Statistical Analysis

Results were analyzed as the means ± standard error. Data were analyzed with one-way analysis of variance (SPSS software) to evaluate statistical significance of the differences. The level of significance was considered at \( P < 0.05 \) and \( P < 0.01 \).

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