The Synthesis and Evaluation of Arctigenin Amino Acid Ester Derivatives

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The use of arctigenin (ARG), a traditional medicine with many pharmacological activities, has been restricted due to its poor solubility in water. Five amino acid derivatives of ARG have been synthesized using glycine, α-alanine, valine, leucine, and isoleucine, which have t-butyloxy carbonyl (BOC) as a protective group. In this study, we examined the effects of removing these protective groups. The results showed that the amino acid derivatives have better solubility and nitrite-clearing ability than ARG. Among the compounds tested, the amino acid derivatives without protective group were the best. Based on these results, ARG and its two amino acid derivatives without protective group (ARG8, ARG10) were selected to evaluate their anti-tumor activity in vivo at a dosage of 40 mg/kg. The results indicated that ARG8 and ARG10 both exhibit more anti-tumor activity than ARG in H22 tumor-bearing mice. The tumor inhibition rates of ARG8 and ARG10 were 69.27 and 43.58%, which was much higher than ARG. Furthermore, the mice treated with these compounds exhibited less damage to the liver, kidney and immune organs compared with the positive group. Furthermore, ARG8 and ARG10 improved the serum cytokine levels significantly compared to ARG.

In brief, this study provides a method to improve the water solubility of drugs, and we also provide a reference basis for new drug development.

Key words arctigenin; amino acid derivative; anti-tumor activity; nitrite-clearing activity; water solubility

Arctigenin (ARG), a natural lignan, is one of the components of Fructus arctii from Arctium lappa L. species. It has been reported to have multiple biological activities, such as anti-tumor,1–7 anti-inflammatory,8–10 and anti-viral11–15 activities. Although ARG exhibits many pharmacological activities in vitro, ARG is too insoluble to be absorbed by the body, which limits its clinical applications.16 Therefore, it is important to modify arctigenin into derivatives with better solubility and higher bioavailability using either chemical or biological methods.

Kudou et al.17 not only synthesized derivatives on the 3 position of arctigenin but also efficiently and flexibly synthesized a variety of derivatives on the 3, 3’ and 4’ positions of arctigenin. Shen et al.18 introduced ethoxy, isopropoxy, 2-methoxyethoxy and (3,4-dimethoxybenzyl) oxy to the 4-OH. However, the solubility of these Poorly soluble drugs in water was not shown to be significantly improved. These reports also did not discuss whether these drugs exhibited biological functions through hydrolysis into raw materials. These drugs also were not able to enter cells through active transport. Amino acid prodrugs are known to be very useful for improving the aqueous solubility of certain drugs.19 The absorption process of amino acids in the small intestine involves active transport with energy dissipation.20,21 For example, Zhang et al.22 designed and synthesized diosgenin derivatives and investigated their antitumor activity. These experiments showed that most compounds had a defined activity. These studies also showed that an amino acid prodrug bound to Silybin resulted in an improvement in water solubility.23 Biasutti et al.24 and Mulholland et al.25 used quercetin as a raw material to make amino acid derivatives to increase the bioavailability.

However, these compounds were synthesized from reactions of the alcohol hydroxyl of primary drugs and the carboxyl of amino acids. The synthesis of the compounds mentioned above involved esterification with alcohol hydroxyl, produced a low yield, and resulted in an unstable compound. Amino acids can actively transport into the human body, and the phenolic OH groups of arctigenin can be further bridged with an appropriate functional group.26 Here, we used the phenolic hydroxyl and the carboxyl of an amino acid under experimental conditions to induce an ester reaction. Because of the phenolic hydroxyl group and benzene ring conjugate, esterification reactions involving phenol are more difficult than those involving aliphatic alcohols.27

It is universally known that nitrite has long been used as a preservative in the food industry.28 What cannot be ignored is that nitrite and secondary amines, tertiary amines or amino compounds react to form strong carcinogenic nitrite amine compounds, which lead to cancers of the digestive system, chronic toxicity and teratogenic risks.29–31 Furthermore, nitrite in the blood stream converts hemoglobin to methemoglobin, thereby interfering with the oxygen transport system of the body.32 Excessive amounts of nitrite in food pose a serious threat to public health. Therefore, the removal of nitrite in vivo is important for cancer prevention.

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors observed clinically,33 and tens of thousands of people die each year as a result. Although these tumors can now be treated with surgery and drug treatment, liver cancer is still difficult to cure. Chemotherapy is one of the effective ways to treat a tumor. However, long term chemotherapy also has many side effects and will do harm to the patient’s body.34 Chemotherapy is used to kill cancer cells that grow quickly. However, because of the drug’s effects
throughout the body, it also affects normal, healthy cells. This is the main cause of the side effects. In recent years, increasingly more natural medicines with good anti-cancer efficacy and low toxicity have been found.\(^\text{35,36}\)

**Results and Discussion**

**Synthesis**

We established a technology for the synthesis of arctigenin amino acid ester, derivatives and we determined that the synthesis of arctigenin–BOC-L-amino acid–1-ethyl-(3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI)–4-dimethylaminopyridine (DMAP) = 1:2:2:0.5), by improving the quality of the reactant (g) and solvent (mL) ratio (1:10), by using the solvent acetonitrile, by maintaining the reaction temperature in an ice water bath, and by using a reaction time of 1–2 h. This method has advantages over the other process, including a higher yield and an easy protocol. This method is also both scientific and reasonable, and we expect it to contribute to the future direction of amino acid esterification. Finally, we identified ARG1, ARG2, ARG3, ARG4 and ARG5. Then, we carried out the experiment of BOC deprotection. Samples (0.2 g) were accurately weighed and then added to 4 mL of anhydrous ethyl acetate solution to be dissolved. Dry hydrogen chloride gas was evenly dispersed through the bottle with anhydrous ethyl acetate, and the reaction took place in an ice water bath for 1 h with TCL detected. Finally, we generated the deprotection compounds ARG6, ARG7, ARG8, ARG9, and ARG10. The synthetic construction of ARG1–10 is shown in Fig. 1.

**Water Solubility of Arctigenin and Its Derivatives**

According to the standards of the National Pharmacopoeia, we examined the water solubility of arctigenin and its derivatives. The results are shown in Table 1. The water solubility of the deprotection compounds was greater than that of arctigenin. The water solubility of the derivatives also improved. Above all, we found that the amino acid derivative compounds ARG6–10 demonstrated increased solubility in water.

**In Vitro Nitrite Clearance Test**

We evaluated the clearance of NO\(^2\) by the derivatives. Due to the low yield of ARG6 and ARG7, we chose ARG8, ARG9 and ARG10 for the nitrite-scavenging test. Furthermore, we investigated the nitrite-scavenging effects of ARG1–5. As shown in Table 2, the nitrite removal ability of the derivative gradually improved with an increase in concentration. At a concentration of 4 mg/mL, the nitrite removal ability increased more than 20% compared with ARG. ARG8 showed the largest increase (30%).

Table 3 shows the nitrite scavenging activity of ARG and its derivatives. The nitrite removal ability of the derivatives was stronger than ARG. ARG8–10 also had a stronger activity than ARG3–5, and their scavenging abilities were similar to VC.

**In Vivo Antitumor Experiments**

The Effects of ARG, ARG8 and ARG10 on Tumor Growth, Thymus Indexes and Spleen Indexes in Mice

The body weights of all of the tumor-bearing mice in all of the groups increased by varying degrees. Compared with the tumor control groups, the weights of the Positive group significantly decreased. The inhibitive effects of cyclophosphamide (CTX), ARG, ARG8 and ARG10 on the growth of the transplanted H\(_{22}\) tumors in mice is shown in Table 4. Our results clearly indicate that the Positive group and the three experimental groups (ARG, ARG8, ARG10) significantly reduced the tumor weights (\(p<0.01\)), and tumor suppression effects of ARG were far less than those of CTX. As shown in Table 4, the ARG8 and ARG10 groups significantly reduced the tumor weights compared with the ARG group (\(p<0.05\) or \(p<0.01\)). We discovered that the tumor growth inhibition rate of ARG8 was comparable to that of CTX. Indeed, the tumor suppression function of ARG8 was almost twice that of ARG.

Compared with the normal group, these two indexes rose significantly in the model group. However, the immune organ

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Table 1. Water-Solubility of ARG and Its Derivatives

| Compound | Water-solubility | Compound | Water-solubility |
|----------|------------------|----------|------------------|
| ARG      | Practically insoluble | ARG6    | Slight soluble  |
| ARG1     | Practically insoluble | ARG7    | Very easily soluble |
| ARG2     | Practically insoluble | ARG8    | Very easily soluble |
| ARG3     | Practically insoluble | ARG9    | Very easily soluble |
| ARG4     | Practically insoluble | ARG10   | Very easily soluble |
| ARG5     | Practically insoluble | | |
Cytokines are important to immune responses and play a pivotal role in fighting against tumor growth. To assess cytokine levels in mice with tumors more than ARG, ARG8 and ARG10 improved the effects on spleen function in the ARG8 and ARG10 groups than in the Positive group, and the highest levels were observed in the ARG8 and ARG10 groups (p<0.01). We also observed that the levels of IL-2 and TNF-α in the ARG8 group were remarkably increased compared with the ARG group (p<0.05). These data indicate that ARG8 and ARG10 might enhance immune function in H22 tumor-bearing mice by increasing cytokine levels.

The Effects of ARG, ARG8 and ARG10 on Hepatic and Renal Function

High levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are usually believed to be a reflection of hepatic disorders, and high creatinine/urea levels are often indicative of kidney malfunction. As shown in Fig. 3, the levels of serum ALT and AST in the ARG, ARG8 and ARG10 groups were all substantially reduced compared to the Positive group (p<0.01). The levels of serum ALT and AST were increased in the Positive group, and the highest levels were observed in the ARG8 and ARG10 groups (p<0.01). We also observed that the levels of IL-2 and TNF-α in the ARG8 group were remarkably increased compared with the ARG group (p<0.05). These data indicate that ARG8 and ARG10 might enhance immune function in H22 tumor-bearing mice by increasing cytokine levels.

The Effects of ARG, ARG8 and ARG10 on Serum Cytokines Levels in Mice

Cytokines are important to immune responses and play a pivotal role in fighting against tumor growth. To assess cytokine secretion associated with the antitumor immunity induced by ARG, ARG8 and ARG10 in H22 tumor-bearing mice, the serum levels of interleukin-2 (IL-2) and tumor necrosis factor (TNF)-α were measured. As shown in Fig. 2, the levels of IL-2 and TNF-α were greatly reduced after CTX treatment compared with the model group (p<0.05 or p<0.01). The levels of IL-2 and TNF-α were significantly higher in the ARG, ARG8 and ARG10 groups than in the Positive group, and the highest levels were observed in the ARG8 and ARG10 groups (p<0.01). The levels of serum ALT and AST in the ARG, ARG8 and ARG10 groups were all substantially reduced compared to the Positive group (p<0.01). Compared with ARG, the levels of serum ALT and AST in the ARG8 and ARG10 groups were closer to normal levels. These results demonstrate that ARG did not have any toxic side effects on the mouse liver, particularly ARG8 and
ARG10. As shown in Fig. 4, the levels of blood urea nitrogen (BUN) and creatinine (CRE) were improved after CTX treatment compared with the model group ($p<0.01$). Compared to the positive group, the levels of serum BUN and CRE were reduced to various degrees after ARG, ARG8 and ARG10 treatment. The levels in the ARG8 and ARG10 groups were close to normal levels. This result indicates that ARG, ARG8 and ARG10 do not have toxic effects on the kidney. ARG8 and ARG10 may even improve renal function in H22 tumor-bearing mice. These results suggest that ARG8 and ARG10 treatment could enhance liver and kidney function more than CTX treatment and change those altered parameters to near normal levels in tumor-bearing mice.

**Conclusion**

In conclusion, we have synthesized a series of arctigenin amino acid derivatives. Furthermore, we found that the water solubility and bioavailability of these derivatives improved. Firstly, we selected the appropriate catalyst. We found that dicyclohexylcarbodiimide (DCC) and DMAP work well as catalysts and that DMAP displayed more catalytic activity than DCC. We also observed that increasing the temperature had no effects on the catalytic activity and that the arctigenin conversion rate was basically stable. When using DCC as the catalyst, white precipitate by-products of $N,N'$-dicyclohexylurea (DCU) were produced, which were difficult to separate from the arctigenin amino acid derivatives. When DMAP was added, the reaction not only esterified rapidly at low temperatures but also resulted in an improved reaction yield. The separation and purification were also easier. Therefore, we chose to use DMAP as the catalyst. We then considered the ratio of the reactants. The esterification reaction is a
remarkable pharmacological activities in vivo tumor-bearing mice. In summary, arctigenin derivatives have proven the bioavailability of active plant ingredients. Antitumor drugs. Our work provides a feasible method to improve the potential of arctigenin and its amino acid derivatives, as well as the immune response in tumor-bearing mice. The levels of cytokines TNF-α and IL-2 were also studied, and the results indicated that ARG8 and ARG10 can markedly increase the serum levels of TNF-α and IL-2. We also conducted an in vivo experiment to study the anti-tumor potential of arctigenin and its amino acid derivatives, as well as the immune response in tumor-bearing mice. The results of the cytokines TNF-α and IL-2 were also studied, and the results indicated that ARG8 and ARG10 can markedly increase the serum levels of TNF-α and IL-2. These results indicate that ARG8 and ARG10 have greater anti-tumor activities in vitro and in vivo, and they also improve immune responses in tumor-bearing mice. In summary, arctigenin derivatives have remarkable pharmacological activities in vitro and in vivo and have potential applications as nitrite scavenging agents and antitumor drugs. Our work provides a feasible method to improve the bioavailability of active plant ingredients.

Experimental

Chemicals were purchased from Sigma-Aldrich, Aladdin, Merck, etc. Column chromatography was performed on a 50 μm YMC, C18 silica gel (Japan), while TLC was performed on a Hg/T23354-2010 silica gel (Qingdao Haiyang Chemical Co., Ltd., China). The purity was determined to be greater than 98.0% by HPLC (Alltech). The structure was identified by comparing the physicochemical and spectroscopic (high-resolution-electrospray ionization-mass spectrum (HR-ESI-MS) and NMR) data. The samples were weighed using an ALI104-type electronic balance (Mettler Toledo, Germany), and a vortex mixing apparatus (The Lindberg Instrument Manufacturing Co., Ltd., Haimen, China) was also used. A RE-52AA-type rotary evaporator (Shanghai Rong Biochemical Instrument Plant, China) was used in the study, and the vacuum conditions were created using a water circulating, multipurpose vacuum pump (Zhengzhou Great Wall Industry and Trade Co., Ltd., China). The temperature was raised and maintained using a HH-4 type thermostatic bath with a digital display (The Jintan City, Jiangsu Province Splendor Instrument Manufacturing Co., Ltd., China). Optical density (OD) values were determined using a SPECTRAMAX 190 Multi-scan Spectrum (The Valley of Molecular Instrument Co., Ltd., Shanghai, China). A QK-250DB-type CNC ultrasonic cleaner was used ( Kunshan Ultrasonic Instrument Co., Ltd., China), and the 96-well plates were obtained from What Instrument Beijing Technology Co., Ltd., China.

Extraction of Arctigenin

Large scale production of arctigenin was performed using the best extraction procedure obtained from the above experiments. A large ultrasonic cleaner (power 40 kHz, acoustical power 2000 W) was used to produce the arctigenin. Approximately 10 kg of pretreated powder (60 mesh) was added to the ultrasonic cleaner and extracted with 125 L of water and 200 g of β-glucosidase. The solution was then sonicated for 1 h at room temperature. After that, ethanol (95%, v/v) was added to the extraction solution to lower the concentration of the extraction (30%, v/v) solution. The solution was then extracted for 12 h and sonicated for 1 h under the same conditions. The extraction solution was filtered, and the remaining material was extracted again with 10 and 8 times the volume of ethanol (30%, v/v). Finally, all of the arctigenin-containing solvent was completely evaporated. A portion of the dried extraction (70 g) was mixed with 105 g of silica gel. The sample was then chromatographed on a silica gel (100–200 mesh) column, and 500 mL of eluent was formulated as a fraction. The elution was performed using a solvent mixture of chloroform and methanol at a 40:1 ratio. In the 12th, 13th, and 14th fractions, canary yellow jellies and crystals appeared after evaporating, and 30 g of pure arctigenin was obtained by recrystallization treatment. This experiment indicates that the preparation procedure is feasible.

A General Procedure for the Preparation of Compounds 1–10

The following reaction conditions were used to produce arctigenin: an amino acid–EDCI–DMAP ratio of 1:2:2:0.5 was used; then the mixture was dissolved in acetonitrile for 1–2 h at 0°C; finally, the mixture was removed under reduced pressure to produce yellow powder. The yellow dope was added to water, washed by stirring, dried, and then concentrated by lyophilization to produce the white crude product. The crude products were chromatographed using silica gel and eluted with acetonitrile–water (55:45) to produce the pure products ARG1–5. We adopted the method using HCl gas to synthesize the deprotection derivatives ARG6–10.

White solid (80%), UV λ_{max} (MeOH) nm: 221, 262. HR-ESI-MS m/z: 529.2315 (Calcd for C_{29}H_{37}O_{9}N: 529.2312). ESI-MS m/z: 547.2 [M+NH_4]^+. 1H-NMR (300 MHz, CDCl_3) δ: 6.97 (d, J=7.8 Hz, 1H), 6.77 (d, J=2.11 Hz, 1H), 6.49 (d, J=2.1 Hz, 1H), 6.54 (dd, J=8.11 Hz, 1H), 6.54 (dd, J=8.1 Hz, 1H), 6.49 (d, J=2.1 Hz, 1H), 1.50 (m, 1H, 2.40, –NH–CH–COO–), 3.91, 4.14 (m, –COOCH_2–), 3.83 (s, 3H), 2.91–2.97 (m, 2H), 4.49 (d, J=2.1 Hz, 1H), 5.09 (m, 1H, 1.40, (–OH–CH–COO–), 3.91, 4.14 (m, –COOCH_3–), 3.83 (s, 3H), 2.91–2.97 (m, 2H), 2.68 (m, 1H, 2.59 (m, 1H), 2.53–2.61 (m, 2H), 1.45 (s, 3H). 13C-NMR (300 MHz, CDCl_3) δ: 136.93, 112.02, 37.32, 64.11, 112.32, 113.42, 149.11, 147.96, 111.56, 122.39, 38.08, 40.90, 71.15, 168.45, 40.97, 157.36, 77.12, 28.23, 55.86, 55.51, 55.77.

White solid (81%), UV λ_{max} (MeOH) nm: 221. HR-ESI-MS m/z: 543.2471 (Calcd for C_{29}H_{37}O_{9}N: 543.2468). ESI-MS m/z: 561.6 [M+NH_4]^+, 1H-NMR (300 MHz, CDCl_3) δ: 6.98
(d, J = 7.8 Hz, 1H), 6.79 (d, J = 8.1 Hz, 1H), 6.76 (d, J = 2.1 Hz, 1H), 6.70 (dd, J = 7.8, 2.1 Hz, 1H), 6.56 (dd, J = 8.1, 2.1 Hz, 1H), 6.51 (d, J = 2.1 Hz), 5.12 (NH), 4.61 (m, –NH–CH–COO–), 3.93, 4.21 (m, –COOCH3–), 3.86 (s, 3H), 3.83 (s, 3H), 3.76 (s, 3H), 2.97–3.00 (m, 2H), 2.70 (m, 1H), 2.60 (m, 1H), 2.54–2.63 (m, 2H), 1.58 (s, 3H), 1.47 (s, 3H). 13C-NMR (300 MHz, CDCl3) δ: 138.41, 113.31, 150.01, 136.82, 124.43, 120.53, 38.13, 49.29, 178.49, 121.51, 118.85, 149.01, 147.86, 111.39, 121.46, 40.95, 34.61, 71.26, 171.58, 46.20, 18.76, 156.04, 67.63, 63.25, 28.31, 28.14, 55.86, 55.81, 55.77.

[(R)-4-((3R,4R)-4-(3,4-Dimethoxybenzyl)-2-oxotetrahydrofuran-3-yl)methyl]-2-methoxyphenyl 2-(tert-Butoxy carbonyl amino)-3-methylpentanoate (ARG3)

White solid (83%), UV λmax (MeOH) nm: 221. HR-ESI-MS m/z: 571.2784 (Calcd for C27H28O12N3: 571.2788)

13C-NMR (300 MHz, CDCl3) δ: 138.41, 113.31, 150.01, 136.82, 124.43, 120.53, 38.13, 49.29, 178.49, 121.51, 118.85, 149.01, 147.86, 111.39, 121.46, 40.95, 34.61, 71.26, 171.58, 46.20, 18.76, 156.04, 67.63, 63.25, 28.31, 28.14, 55.86, 55.81, 55.77.

[(R)-4-((3R,4R)-4-(3,4-Dimethoxybenzyl)-2-oxotetrahydrofuran-3-yl)methyl]-2-methoxyphenyl 2-(tert-Butoxy carbonyl amino)-4-methylpentanoate (ARG4)

White solid (71%), UV λmax (MeOH) nm: 221. HR-ESI-MS m/z: 585.2941 (Calcd for C27H28O12N3: 585.2938)

13C-NMR (300 MHz, CDCl3) δ: 138.41, 113.27, 151.05, 136.80, 122.52, 120.51, 34.62, 46.39, 78.49, 130.20, 113.29, 149.18, 148.02, 111.63, 121.47, 38.15, 40.99, 71.21, 170.41, 58.62, 31.33, 18.99, 17.22, 155.75, 77.17, 28.39.

[(S)-4-((3R,4R)-4-(3,4-Dimethoxybenzyl)-2-oxotetrahydrofuran-3-yl)methyl]-2-methoxyphenyl 2-(tert-Butoxy carbonyl amino)-4-methylpentanoate (ARG4)

White solid (71%), UV λmax (MeOH) nm: 221. HR-ESI-MS m/z: 585.2941 (Calcd for C27H28O12N3: 585.2938)

13C-NMR (300 MHz, CDCl3) δ: 138.41, 113.27, 151.05, 136.80, 122.52, 120.51, 34.62, 46.39, 78.49, 130.20, 113.29, 149.18, 148.02, 111.63, 121.47, 38.15, 40.99, 71.21, 170.41, 58.62, 31.33, 18.99, 17.22, 155.75, 77.17, 28.39.

[(S)-4-((3R,4R)-4-(3,4-Dimethoxybenzyl)-2-oxotetrahydrofuran-3-yl)methyl]-2-methoxyphenyl 2-(tert-Butoxy carbonyl amino)-4-methylpentanoate (ARG5)

White solid (80%), UV λmax (MeOH) nm: 221. HR-ESI-MS m/z: 585.2941 (Calcd for C27H28O12N3: 585.2938)

13C-NMR (300 MHz, CDCl3) δ: 138.41, 113.27, 151.05, 136.80, 122.52, 120.51, 34.62, 46.39, 78.49, 130.20, 113.29, 149.00, 147.83, 111.38, 121.45, 38.11, 41.80, 71.26, 171.60, 52.19, 40.94, 24.78, 22.91, 21.92, 161.25, 79.88, 28.31, 56.06, 55.87, 55.76.

[(2R)-4-((3R,4R)-4-(3,4-Dimethoxybenzyl)-2-oxotetrahydrofuran-3-yl)methyl]-2-methoxyphenyl 2-(tert-Butoxy carbonyl amino)-3-methylpentanoate (ARG5)

White solid (80%), UV λmax (MeOH) nm: 221. HR-ESI-MS m/z: 585.2941 (Calcd for C27H28O12N3: 585.2938)

13C-NMR (300 MHz, CDCl3) δ: 138.41, 113.27, 151.05, 136.80, 122.52, 120.51, 34.62, 46.39, 78.49, 130.20, 113.29, 149.00, 147.83, 111.38, 121.45, 38.11, 41.80, 71.26, 171.60, 52.19, 40.94, 24.78, 22.91, 21.92, 161.25, 79.88, 28.31, 56.06, 55.87, 55.76.

[(2R)-4-((3R,4R)-4-(3,4-Dimethoxybenzyl)-2-oxotetrahydrofuran-3-yl)methyl]-2-methoxyphenyl 2-(tert-Butoxy carbonyl amino)-3-methylpentanoate (ARG5)

White solid (80%), UV λmax (MeOH) nm: 221. HR-ESI-MS m/z: 585.2941 (Calcd for C27H28O12N3: 585.2938)

13C-NMR (300 MHz, CDCl3) δ: 138.41, 113.27, 151.05, 136.80, 122.52, 120.51, 34.62, 46.39, 78.49, 130.20, 113.29, 149.00, 147.83, 111.38, 121.45, 38.11, 41.80, 71.26, 171.60, 52.19, 40.94, 24.78, 22.91, 21.92, 161.25, 79.88, 28.31, 56.06, 55.87, 55.76.
(2R)-4-(((3R,4R)-4-(3,4-Dimethoxybenzyl)-2-oxotetrahydrofuran-3-yl)(methyl))-2-methoxyphenyl 2-Amino-3-methylpentanoate Hydrochloride (ARG10)

White solid (71%), UV λ_max (MeOH) nm: 221. HR-ESI-MS m/z: 521.2184 (Caled for C_{27}H_{35}O_{7}NCl: 521.2180). ESI-MS m/z: 539.5 [M+NH_4]^+. 1H-NMR (300 MHz, CDCl_3) δ: 6.76 (d, J=2.1 Hz, 1H), 7.28 (d, J=7.8 Hz, 1H), 6.64 (dd, J=8.1, 1.8 Hz, 1H), 2.81–2.93 (m, 2H), 2.73 (m, 1H), 6.49 (d, J=2.1 Hz, 1H), 7.02 (d, J=8.1 Hz, 1H), 6.56 (dd, J=8.4, 1.2 Hz, 1H), 2.57–2.45 (m, 2H), 2.50 (m, 1H), 4.13–4.25 (m, 2H), 3.84 (s, 3H), 4.25 (m, 1H), 2.05 (m, 1H), 1.49 (m, 1H), 0.94 (s, 3H), 1.15 (d, J=6.9 Hz, 3H), 5.11 (NH). 13C-NMR (300 MHz, CDCl_3) δ: 137.36, 112.23, 150.72, 137.78, 121.55, 120.69, 36.61, 46.38, 178.31, 130.21, 113.41, 149.10, 147.95, 111.65, 122.75, 38.14, 41.07, 71.27, 56.11, 55.95, 55.91, 166.54, 57.45, 34.72, 25.32, 11.63, 14.63.

Solubility Experiments Arctigenin and its derivatives (1 mg), in strict accordance with the National Pharmacopoeia, were added to water at a temperature of 25±2°C and vibration waves were applied every 5 min for 30 s. The samples were observed after 30 min, and the dissolution was considered complete when there were no longer any visible solute particles or droplets.

Nitrite Scavenging Activity (NSA) In this experiment, V_C was used as a positive control to compare to the samples or droplets. The required amounts of phospholipids (8 mg/mL), cholesterol (24 mg/mL), ARG or derivatives (compounds 1–5, ranging from 0.125 to 4 mg/mL) were dissolved in absolute dichloromethane (5 mL) by stirring. The obtained organic solution was then recovered under reduced pressure. Buffer solution (5 mL, pH=3) was then added to the liposomal suspension, which was stored at 4°C. However, V_C and compounds 8–10 (ranging from 0.25 to 8 mg/mL) were dissolved in the buffer solution directly, rather than being dissolved in the solution made of liposomes. Buttes without ARG or derivatives were considered to be blank liposomes. The sample (40 µL) was added to 20 µL of 1 mL NaNO_2 and mixed. The mixture was then adjusted to a volume of 40 µL with distilled water and adjusted with disodium hydrogen phosphate and citric acid buffer solution to a pH of 3. The reaction solution was incubated at 37°C for 1 h, and then 40 µL of 0.4% sulfuric acid was added to 20 µL of the reaction mixture solution. The reaction mixture was shaken thoroughly for 5 min, and 20 µL of 0.2% N-1-naphthyl oxalic acid amine hydrochloride and 120 µL distilled water was mixed into the reaction. The whole process was carried out for 15 min at room temperature, and the absorbance at 490 nm was measured. A blank was prepared according to the method described above. However, 40 µL of buffer solution was added instead of the sample. The results were interpreted using the following formula: NSA (%)=[(A−B)/A]×100%, with A as the absorbance of the blank group and B as the absorbance of the sample group.

Animals and Cell Lines Specific Pathogen Free (SPF) grade, male ICR mice with body weights ranging from 19 to 21 g were obtained from the Laboratory Animal Testing Center of Jilin Province. They were raised in an aseptic environment (23±2°C, 55±5% humidity) on a 12-h light/12-h dark cycle with a standard pellet diet and water supplied ad libitum throughout the experimental period. The animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of China Academy of Chinese Medical Sciences. H_22 hepatocarcinoma cells were purchased from the Institute of Biochemistry and Cell Biology, CAS.

In Vivo Anti-tumor Experiments The experiments were conducted under aseptic condition. Ascites fluid was taken from the hepatoma H_22-bearing mice and diluted with normal saline into a suspended solution at a concentration of 1×10^6 cells/mL. To set up the ascitic tumor-bearing model, 0.2 mL of the cell suspension (1×10^6 cells/mouse) was subcutaneously injected into the shoulder of the right limb of each mouse. The ICR mice were divided into the following five groups 24 h after tumor inoculation with the H_22 cells: a normal group, a model group, a CTX group, an Arctigenin group, an ARG8 group, and an ARG10 group. The model group and the normal group were treated orally with equal amounts of normal saline. The positive control group received CTX at a dosage of 25 mg/kg by intraperitoneal injection. The arctigenin group, the ARG8 group and the ARG10 group all received an oral dosage of 40 mg/kg. The mice in each group were treated continuously for 14 d. The tumor sizes were measured and the weights of the mice were recorded before and after each drug treatment. The mice were sacrificed by cervical dislocation 24 h after the last administration, and blood samples were collected from the eyes. The tumors, spleens, and thymuses of the mice were taken out and weighed using an electronic balance. The tumor inhibition rate was calculated as follows: inhibitory rate=[(the tumor weight of the model group)–(the tumor weight of the treatment group)]/(the tumor weight of the model group)×100%. The organ indexes of the spleens and thymuses were calculated as follows: organ index (%)=[(average weight of organ/average body weight)]×100%.

Measurement of AST, ALT, CRE and BUN AST and ALT are clinical indexes of liver function that are used to judge whether the liver is damaged, and BUN and CRE are used to judge whether the kidney is damaged. Their levels were analyzed using a commercially available reagent kit according to the instructions.

Measurement of Cytokines The serum levels of TNF-α and IL-2 were analyzed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions.

Statistical Analysis Statistical analysis was performed using SPSS version 16.0 (SPSS, Chicago, IL, U.S.A.). The data are expressed as the mean±standard deviation (S.D.), and significant differences were assessed using Student's t-test. p<0.05 was considered statistically significant.

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Conflict of Interest The authors declare no conflict of interest.

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