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Synthesis and evaluation of asiatic acid derivatives as anti-fibrotic agents: Structure/activity studies

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ABSTRACT

Fibrotic diseases are characterized by the over-accumulation of fibrous components in the extracellular matrix and the liver, which can lead to liver cirrhosis. Current treatment options cannot reverse or halt liver fibrosis, motivating a search for newer treatment options. Previously, we showed that asiaticoside, a bioactive triterpene glycoside from *Centella asiatica*, has anti-fibrotic properties. Here, the aglycone asiatic acid was chemically modified, and these derivatives were evaluated for their potential as anti-fibrotic agents. The data obtained from *in vivo* testing of these compounds in a rodent CCl_4-induced liver injury model are discussed. The information obtained from these studies may be useful in the design of novel anti-fibrotic agents.

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1. Introduction

Cirrhosis, a pathological condition characterized by liver dysfunction due to excessive fibrosis, is the consequence of chronic liver diseases of any etiology and is the major determinant of morbidity and mortality in liver patients. In advanced stage cirrhosis, a liver transplant is currently the only treatment option. Therefore, there is a pressing need for the development of new treatment options that can prevent, halt or reverse hepatic fibrosis [1]. Hepatic fibrosis is characterized by increased proliferation and excessive deposition of extracellular matrix (ECM) [2,3]. Recent investigations have unraveled the dynamic pathophysiological mechanisms underlying hepatic fibrosis and have identified several potential targets [4,5]. In animal models, interference with TGF-β receptor signaling has been shown to modulate fibrosis. Anti-inflammatory agents and antioxidants are being pursued as treatment options for liver fibrosis. According to Bataller and Brenner [6], the identification of new agents effective at reversing or halting fibrosis will aid the search for potential treatment options.

Centella asiatica (Apiaceae) is widely used in the treatment of skin inflammation resulting from wounds, burns, or other injuries. Extracts of *C. asiatica* are widely used in natural medicines including ointments, dentifrices and cosmetics. The main bioactive constituents of this medicinal plant include triterpenoids such as madecassoside, asiaticoside, madecassic acid and asiatic acid [7,8]. Asiaticoside, a triterpene glycoside, has been reported to possess wound healing [9,10] and antidepressant properties [11,12]. Previously, we demonstrated that asiaticoside also displays anti-fibrotic properties. Because asiaticoside is a glycosidic derivative of asiatic acid, we hypothesized that the pharmacophore responsible for the anti-fibrotic activity is the asiatic acid moiety. The present study, therefore, investigates the anti-fibrotic effects of asiatic acid and its non-glycosidic derivatives on liver fibrosis. Further, the molecular mechanisms underlying the anti-fibrotic effects of these derivatives were investigated in *in vivo* models.

2. Experimental

2.1. Chemistry

Asiatic acid retains the ursane skeleton of triterpenoids and is functionalized at four positions: hydroxyls at positions C-2α, C-3β and C-23, and a carboxylic acid functionality at position C-28. To evaluate the potential of asiatic acid as an anti-fibrotic agent, we synthesized several derivatives of it. These derivatives modify the carboxylic acid with additional functional groups that may interact with potential intra-cellular targets via hydrogen bonds. To bring about modifications to the C-28 carboxylic acid, the acid was esterified with ethyl bromoacetate, following which the ethyl

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ester was modified to various primary and secondary amides. A second set of analogues explored the effect of structural modification to the A-ring of the triterpenoid. For this, the 1,3-dihydroxy moiety on the A-ring was reacted with aryl and alkyl ketones to generate substituted 1,3-dioxane derivatives.

*2.1.1. Synthesis of the amides **4a**, **4b**, **4c**, **4d** and **4e** (Scheme 1)*

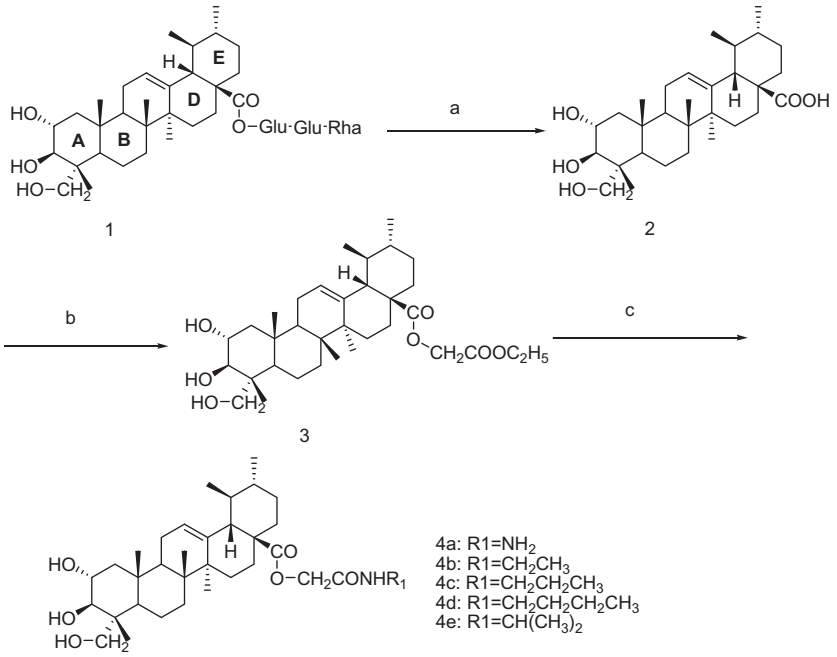
The asiatic acid derivatives **4a**, **4b**, **4c**, **4d** and **4e** containing an amide group attached to pendant carboxylic acid at C-28 were synthesized in 3 steps as shown in Scheme 1. First, asiaticoside **1** was saponified to generate free asiatic acid **2**. Next, nucleophilic attack by the carboxylate of asiatic acid **2** on the halogenated-carbon of ethyl bromoacetate was initiated in the presence of K_2CO_3, Et_3N and KI, following a synthetic method developed in our laboratory, to obtain hydroxyl acetate derivative **3**. The structure of **3** was confirmed by comparing its IR and ^1H NMR spectra with those of analogous compounds synthesized previously. The primary amide **4a** was generated by the reaction of **3** with ammonia in ethanol. The structure of **4a** was confirmed by analysis of its IR, proton and carbon NMR data. Finally, compounds **4b**, **4c**, **4d** and **4e** were synthesized by methods similar to the one used in the synthesis of **4a**.

*2.1.2. Synthesis of the 1,3-dioxane derivatives **5a**, **5b**, **5c**, **5d**, **5e** and **5f**. (Scheme 2)*

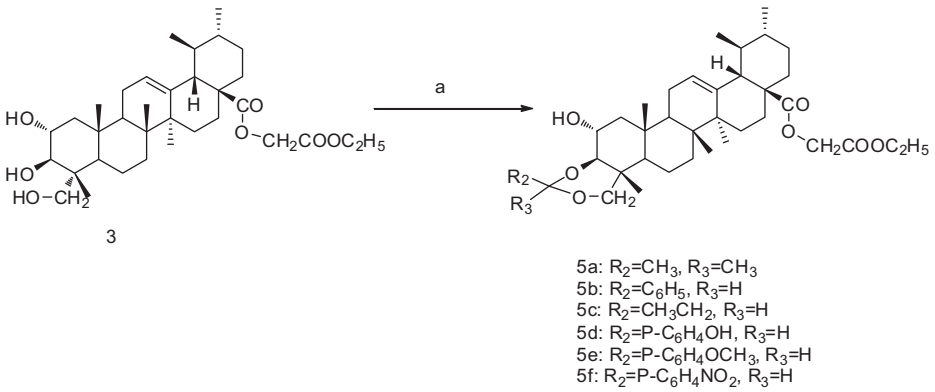
To evaluate the effect of structural modifications on the A-ring, we synthesized the new compounds **5a**, **5b**, **5c**, **5d**, **5e** and **5f**. The synthesis of these compounds involved the condensation of asiatic acid with ketones or aldehydes to generate a cyclic acetal. The 1,3-dioxane containing compound **5a** was obtained when the 1,3-dihydroxyl moieties of asiatic acid (hydroxyls on C-3 and C-23) and acetone were reacted in the presence of DMF and $HClO_4$. The structure of **5a** was determined by a comparison of its IR and NMR spectral data with those of **3**. The compounds **5b**, **5c**, **5d**, **5e** and **5f** were essentially prepared by similar routes.

2.2. Biological evaluation

Female Sprague–Dawley (SD) rats weighting 150–190 g were used in these studies. The animals were kept under controlled conditions and were fed with solid stock food and tap water. The rats were randomized by weight and grouped into a model group (n = 12, 6 females and 6 males) and a normal control group (n = 12, 6 females and 4 males). Rats in the model group



Scheme 1. Reagents: (a) (1) NaOH, (2) HCl; (b) $BrCH_2COOC_2H_5$, K_2CO_3, Et_3N, KI; (c) CH_3CH_2OH, NH_2R_1.



Scheme 2. Reagents: (a) DMF, $HClO_4$, R_2COR_3.

were administered 0.5 mL/100 g gavage doses of the 12 asiatic acid derivatives as CMC (carboxy methyl cellulose) suspensions once a day for 6 consecutive weeks. The normal control group was simultaneously administered the vehicle control–CMC solution–through the oral gavage route. To induce liver cirrhosis and fibrosis, intraperitoneal injection of the normal control group began with 0.2 mL/100 g 20% canola oil two times a week for six weeks; the remaining rats were injected with an equivalent amount of 20% carbon tetrachloride solution two times a week for six weeks (i.p.). Rats in the normal control group were injected i.p. with 0.2 mL/100 g of oil alone two times a week for six weeks. (By 4–5 weeks after the initial i.p. injection of the CCl_4:oil solution, we observed an increase in the number of deaths of the treated rats, and hence, the injection schedule was adjusted from twice a week to once per week for 6 weeks.) The groups of SD rats were weighed once every 7 days and weights were measured 12 h post-fasting. Twenty-four hours after the last drug injection, blood was extracted from the femoral vein, liver clippings were collected and both the samples were submitted for further analysis.

Statistical analysis was performed using the SPSS 17 software, and the experimental data are presented as the mean ± SEM. Using a single factor analysis of variance, $p \leqslant 0.05$ was considered to be statistically significant.

During the course of the experiment, the body weight of most rats increased, however a few rats showed emaciation, with relative emaciation status measured by the occurrence of thin stools and lack of food consumption. In the two weeks following the initial i.p. injection of the CCl_4:oil solution, most rats appeared apathetic, consumed less drinking water and exhibited loss of appetite. After 4–5 weeks after the initial i.p. injection of the CCl_4:oil solution, we observed an increase in the number of deaths of the treated rats, and hence, the injection schedule was adjusted from twice a week to once per week for 6 weeks. These results are shown in Table 1.

3. Results and discussion

As shown in Table 1 (entries **5a**, **5d**, **5b**, **4d**, **4c**, **5f**), the asiatic acid derivatives significantly decreased serum aspartate aminotransferase (AST) activity compared to that in the control group. Some of these compounds (Table 1, entries **4d**, **4a**, **4c**, **5f**) also significantly reduced serum alanine aminotransferase (ALT) activity when compared to the control group. These results suggest that these asiatic acid derivatives are effective at protecting the liver from CCl_4-induced damage. The data presented here may be useful in the design of novel anti-fibrotic agents for hepatic fibrosis. From the results of the pharmacological experiment we can see that derivatives **4a**–**4e** with alkanes (C = 3 or 4) have better pharmacological activity. Moreover, the larger number of carbon atoms, the better the pharmacological activity. For example, compounds **4c** and **4e** are prior to **4a** (see Fig. 1). However, it is important to note that it does not mean the more carbon atoms the better. The reason for this phenomenon is most likely because alkanes are lipophilic, hence allowing compounds **4a**–**4e** to more easily penetrate through the blood lipid barrier of target tissues and cells.

Derivatives **5a**–**5f** with a benzene ring have better pharmacological activity. Moreover, the electron withdrawing group in the benzene ring can further enhance the pharmacological effects of the derivatives. Because there is no conjugated structure in the lead compound molecular asiatic acid, its molecular structure is flexible and its electron cloud is scattered. However, the conjugated structure of the benzene ring and the electron withdrawing group on it impact the electron cloud density of

Table 1
Effects of asiatic acid derivatives on CCl_4-induced chronic liver fibrosis model in rats.

Category	Dose (mg/kg)	Total bilirubin (μmol/L)	Bilirubin direct (μmol/L)	Indirect bilirubin (μmol/L)	AST (IU/L)	ALT (IU/L)	AST/ALT	Liver coefficient	Number of animals/ Deaths
Model control group	8	2.38 ± 0.87	2.13 ± 0.87	0.25 ± 0.096	917.20 ± 313.98$^{\Delta\Delta}$	463.30 ± 114.36$^{\Delta}$	2.00 ± 0.26	0.041 ± 0.0018	12/4
Normal control group	8	1.15 ± 0.10	0.72 ± 0.05	0.43 ± 0.056	172.00 ± 12.44	76.83 ± 4.94	2.25 ± 0.67	0.043 ± 0.0039	12/1
5a group	8	1.38 ± 0.22	0.85 ± 0.11	0.43 ± 0.120	257.00 ± 15.71*	202.67 ± 72.71	1.95 ± 0.43	0.045 ± 0.0029	12/4
5d group	8	1.17 ± 0.22	0.72 ± 0.15	0.45 ± 0.096	291.83 ± 41.83*	130.00 ± 36.19	2.54 ± 0.27	0.041 ± 0.0021	12/3
5b group	8	1.23 ± 0.14	0.73 ± 0.11	0.50 ± 0.052	294.50 ± 83.00*	172.67 ± 64.32	2.06 ± 0.29	0.045 ± 0.0029	12/3
4d group	8	1.15 ± 0.25	0.95 ± 0.22	0.20 ± 0.052	214.00 ± 26.78*	88.67 ± 5.39*	2.39 ± 0.20	0.046 ± 0.0029	12/3
4b group	8	1.37 ± 0.16	0.88 ± 0.13	0.48 ± 0.054	334.17 ± 67.39	217.17 ± 78.49	2.06 ± 0.31	0.042 ± 0.0024	12/4
4e group	8	1.37 ± 0.15	0.78 ± 0.10	0.58 ± 0.094	584.00 ± 247.11	510.±222.76	1.75 ± 0.41	0.045 ± 0.0047	12/3
4a group	8	1.17 ± 0.15	0.68 ± 0.10	0.48 ± 0.070	354.33 ± 56.15	103.17 ± 20.18*	3.77 ± 0.70*	0.035 ± 0.0016	12/2
4c group	8	1.48 ± 0.25	1.03 ± 0.18	0.45 ± 0.085	214.50 ± 27.68*	81.33 ± 11.74*	2.85 ± 0.42	0.038 ± 0.0024	12/3
5e group	8	1.72 ± 0.33	1.17 ± 0.22	0.55 ± 0.120	487.17 ± 103.92	170.17 ± 31.47	3.14 ± 0.83	0.087 ± 0.0047	12/2
5c group	8	1.40 ± 0.14	0.88 ± 0.13	0.52 ± 0.060	435.83 ± 142.40	278.00 ± 123.12	2.27 ± 0.39	0.038 ± 0.0034	12/3
5f group	8	1.97 ± 0.50	1.40 ± 0.39	0.57 ± 0.120	260.17 ± 43.40*	109.00 ± 25.47*	2.54 ± 0.26	0.037 ± 0.0034	12/4
Asiatic acid group	8	1.53 ± 0.35	1.08 ± 0.32	0.45 ± 0.092	187.83 ± 11.09*	79.67 ± 6.85*	2.43 ± 0.22	0.04 ± 0.0033	12/1

$^{\Delta\Delta}$ <0.01; Compared with normal control group.
$^{\Delta}$ <0.05; Compared with normal control group.
* $P < 0.05$; compared with the model group.

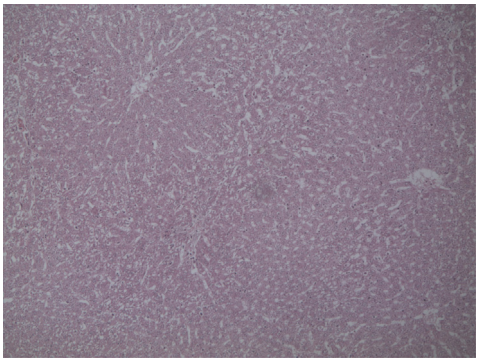


Figure control rat liver H&E×100

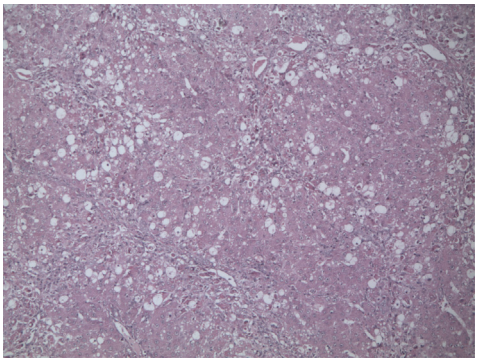


Figure CCl_4-induced liver H&E×100

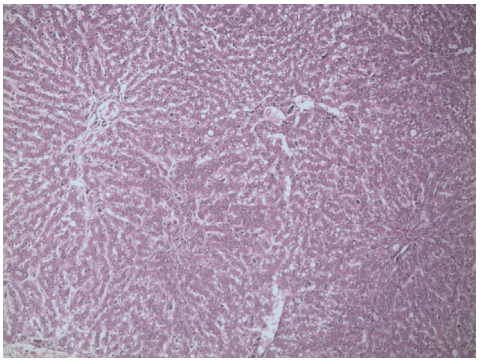


Figure 5d-1 H&E×100

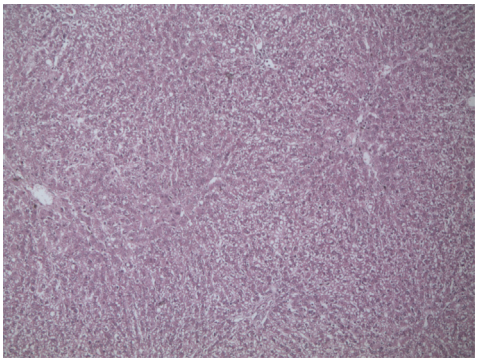


Figure 4d-2 H&E×100

Fig. 1. All experiments were performed in duplicate, and the photographs were taken at 100× magnification.

the lead compound markedly. In a word, the lipotrophy and electron cloud density of the derivatives are relevant to their pharmacological activity.

4. Experimental

4.1. Chemistry

Melting points were recorded with a Micro melting point tester and are uncorrected. ^{1}H NMR spectra were acquired using a Bruker AVII-400 spectrometer (400 MHz). Infrared spectra were recorded using a Perkin–Elmer 16PC-FT spectrometer. Silica gel column chromatography was carried out using GF254 (300–400 mesh) silica gel. High-resolution mass spectra were recorded on a Bruker Daltonics ESI-BioTOF Q spectrometer.

*4.1.1. (1S,2R,4aS,6aS,6bR,9R,10R,11R,12aR,14bS)-2-Hydrazinyl-2-oxoehyl10,11-dihydroxy-9-(hydroxymethyl)-1,2,6a,6b,9,12a-hexamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-icosahydropicene-4a-carboxylate (**4a**)*

First, asiaticoside **1** was saponified to generate free asiatic acid **2**. Next, nucleophilic attack by the carboxylate of asiatic acid 2 on the halogenated carbon of ethyl bromoacetate was initiated in the presence of K_2CO_3, Et_3N and KI, following a synthetic method developed in our laboratory, to obtain hydroxyl acetate derivative **3**. The primary amide **4a** was generated by the reaction of **3** with ammonia in ethanol and hydrazine. θ_{mp} 62–63 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.78 (s, 1H), 8.36 (s, 1H), 5.26 (s, 2H), 4.93 (dd, J_1 = 16.4 Hz, J_2 = 46.4 Hz, 2H), 4.61 (dd, J_1 = 15.2 Hz, J_2 = 20.8 Hz, 1H), 3.73–3.79 (m, 2H), 3.67 (d, J = 10.0 Hz, 2H), 3.42 (t, J = 8.0 Hz, 3 H), 2.29 (d, J = 11.2 Hz, 1H), 2.22 (d, J = 11.2 Hz, 1H), 2.14 (s, 2H), 1.98 (s, 3H), 1.91 (s, 3H), 1.81 (s, 6 H), 1.26 (s, 2H), 21.09 (s, 3H), 1.03 (d, J = 6.4 Hz, 3H), 0.79 (s, 3H), 0.70 (s, 3H). HRMS (ESI) m/z calcd for $C_{32}H_{52}N_2O_6$ $[M+H]^+$ 561.3904, found 561.3911.

*4.1.2. (1S,2R,4aS,6aS,6bR,9R,10R,11R,12aR,14bS)-2-(Ethylamino)-2-oxoethyl10,11-dihydroxy-9-(hydroxymethyl)-1,2,6a,6b,9,12a-hexamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-icosahydropicene-4a-carboxylate (**4b**)*

Compound **4b** was prepared similarly according to the procedure of **4a**. θ_{mp} 62–65 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.19 (s, 1H), 5.26 (s, 1H), 4.68 (d, J = 15.6 Hz, 1H), 4.29 (d, J = 15.6 Hz, 1H), 3.73–3.77 (m, 1H), 3.67 (d, J = 10.4 Hz, 1H), 3.42 (d, J = 10.8 Hz, 2H), 3.29–3.39 (m, 2H), 2.23 (d, J = 11.2 Hz, 1H), 1.93 (d, J = 10.8 Hz, 1H), 1.46–1.49 (m, 1H), 1.32–1.42 (m, 4H), 1.31 (s, 1H), 1.28 (s, 1H), 1.25 (s, 1H), 1.19 (t, J = 7.2 Hz, 3H), 1.10 (s, 3H), 1.01 (s, 3H), 0.97 (s, 3H), 0.96 (s, 1H), 0.88 (s, 3H), 0.87 (s, 3H), 0.69 (s, 3H). HRMS (ESI) m/z calcd for $C_{34}H_{55}NO_6$ $[M+H]^+$ 574.4108, found 574.4142.

*4.1.3. (1S,2R,4aS,6aS,6bR,9R,10R,11R,12aR,14bS)-2-oxo-2-(Propylamino)ethyl10,11-dihydroxy-9-(hydroxymethyl)-1,2,6a,6b,9,12a-hexamethyl 1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-icosahydropicene-4a-carboxylate (**4c**)*

Compound **4c** was prepared similarly according to the procedure of **4a**. θ_{mp} 68–70 °C. ^{1}H NMR 400 MHz, $CDCl_3$) δ 6.20 (s, 1H), 5.26 (s, 1H), 4.66 (d, J = 15.6 Hz, 1H), 4.32 (d, J = 15.6 Hz, 1H), 3.73–3.79 (m, 1H), 3.69 (d, J = 10.0 Hz, 1H), 3.42 (t, J = 10.0 Hz, 2H), 3.20–3.27 (m, 1H), 3.29–3.36 (m, 1H), 2.22 (d, J = 11.2 Hz, 1H), 1.94 (d, J = 10.8 Hz, 1H), 1.63 (d, J = 4.8 Hz, 1H), 1.61 (d, J = 7.6 Hz, 1H), 1.56 (t, J = 7.2 Hz, 2H), 1.52 (d, J = 8.0 Hz, 2H), 1.46–1.49 (m, 1H), 1.32–1.42 (m, 4H), 1.31 (s, 1H), 1.28 (s, 1H), 1.25 (s, 1H), 1.10 (s, 3H), 1.02 (s, 1H), 0.97 (s, 3H), 0.96 (s, 3H), 0.93 (s, 1H), 0.90 (s, 3H), 0.88 (d, J = 6.4 Hz, 3H), 0.70 (s, 3H). HRMS (ESI) m/z calcd for $C_{35}H_{57}NO_6$ $[M+H]^+$ 588.4186, found 588.4139.

*4.1.4. (1S,2R,4aS,6aS,6bR,9R,10R,11R,12aR,14bS)-2-(Butylamino)-2-oxoethyl10,11-dihydroxy-9-(hydroxymethyl)-1,2,6a,6b,9,12a-hexamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-icosahydropicene-4a-carboxylate (**4d**)*

Compound **4d** was prepared similarly according to the procedure of **4a**. θ_{mp} 60–61 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.18 (s, 1H), 5.26 (s, 1H), 4.66 (d, J = 15.6 Hz, 1H), 4.32 (d, J = 15.6 Hz, 1H), 3.73–3.79 (m, 1H), 3.69 (d, J = 10.8 Hz, 1H), 3.42 (t, J = 11.2 Hz, 2H), 3.32–3.38 (m, 1H), 3.24–3.31 (m, 1H), 2.22 (d, J = 11.2 Hz, 1H), 2.02 (t, J = 5.6 Hz, 1H), 1.99 (d, J = 4.4 Hz, 1H), 1.94 (dd, J_1 = 2.0 Hz, J_2 = 3.2 Hz, 1H), 1.74 (s, 1H), 1.60–1.67 (m, 2H), 1.54–1.56 (m, 1H), 1.52 (d, J = 8.0 Hz, 2H), 1.46–1.49 (m, 1H), 1.32 (d, J = 3.6 Hz, 1H), 1.28 (s, 1H), 1.26 (s, 1H), 1.10 (s, 3H), 1.02 (s, 3H), 0.97 (s, 3H), 0.95 (s, 3H), 0.93 (s, 1H), 0.92 (s, 3H), 0.88 (d, J = 6.4 Hz, 3H), 0.70 (s, 3H). HRMS (ESI) m/z calcd for $C_{36}H_{59}NO_6$ $[M+H]^+$ 602.4342, found 602.4318.

*4.1.5. (1S,2R,4aS,6aS,6bR,9R,10R,11R,12aR,14bS)-2-(Isopropylamino)-2-oxoethyl10,11-dihydroxy-9-(hydroxymethyl)-1,2,6a,6b,9,12a-hexamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-icosahydropicene-4a-carboxylate (**4e**)*

Compound **4e** was prepared similarly according to the procedure of **4a**. θ_{mp} 69–72 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.95 (d, J = 7.6 Hz, 1H), 5.26 (s, 1H), 4.57 (d, J = 15.6 Hz, 1H), 4.33 (d, J = 15.6 Hz, 1H), 4.10–4.19 (m, 1H), 3.76 (td, J_1 = 4.4 Hz, J_2 = 10.4 Hz, 1H), 3.68 (d, J = 10.4 Hz, 1 H), 3.42 (t, J = 8.4 Hz, 2H), 2.22 (s, 1H), 2.18 (d, J = 5.6 Hz, 1H), 1.90–2.12 (m, 6H), 1.74 (d, J = 9.2 Hz, 3H), 1.60–1.68 (m, 3H), 1.46–1.57 (m, 3H), 1.28–1.39 (m, 6H), 1.25 (s, 2H), 1.20 (t, J = 6.4 Hz, 6H), 1.10 (s, 3H), 1.02 (s, 3H), 0.96 (d, J = 6.4 Hz, 3H), 0.90 (s, 3H), 0.88 (d, J = 6.4 Hz, 3H), 0.70 (s, 3H). HRMS (ESI) m/z calcd for $C_{35}H_{57}NO_6$ $[M+H]^+$ 588.4265, found 588.4301.

*4.1.6. (4aR,6aR,6bS,8aS,11R,12S,12aS,14bR,16R,16aR)-2-Ethoxy-2-oxoethyl16-hydroxy-2,2,4a,6a,6b,11,12,14b-octamethyl-4a,4b,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b,15,16,16a-icosahydro-4H-piceno[3,4-d][1,3]dioxine-8a-carboxylate (**5a**)*

The 1,3-dioxane containing compound **5a** was obtained when the 1,3-dihydroxyl moiety of asiatic acid (hydroxyls on C-3 and C-23) and acetone were reacted in the presence of DMF and HClO4. θ_{mp} 80–81 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.26 (t, J = 3.2 Hz, 1H), 4.57 (dd, J_1 = 16 Hz, J_2 = 38 Hz, 2H), 4.19 (q, J = 7.2 Hz, 2H), 3.78 (td, J_1 = 4.4 Hz, J_2 = 10.0 Hz, 1H), 3.49 (dd, J_1 = 10.8 Hz, J_2 = 17.6 Hz, 2H), 3.32 (d, J = 9.6 Hz, 1H), 2.26 (d, J = 9.2 Hz, 1H), 2.05 (t, J = 4.8 Hz, 1H), 1.95 (dd, J_1 = 3.6 Hz, J_2 = 8.8 Hz, 2H), 1.69–1.83 (m, 4H), 1.62 (t, J = 8.8 Hz, 1H), 1.45 (d, J = 3.6 Hz, 6H), 1.51 (td, J_1 = 3.2 Hz, J_2 = 13.2 Hz, 2H), 1.31–1.43 (m, 3H), 1.27 (t, J = 7.2 Hz, 3H), 1.10 (s, 3H), 1.07 (s, 3H), 1.03 (s, 3H), 0.95 (d, J = 6.0 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H), 0.72 (s, 3H). HRMS (ESI) m/z calcd for $C_{37}H_{58}O_7$ $[M+H]^+$ 615.4262, found 615.4232.

*4.1.7. (2R,4aR,6aR,6bS,8aS,11R,12S,12aS,14bR,16R,16aR)-2-Ethoxy-2-oxoethyl16-hydroxy-4a,6a,6b,11,12,14b-hexamethyl-2-phenyl-4a,4b,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b,15,16,16a-icosahydro-4H-piceno[3,4-d][1,3]dioxine-8a-carboxylate (**5b**)*

Compound **5b** was prepared similarly according to the procedure of **5a**. θ_{mp} 25–30 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, J = 7.6 Hz, 2H), 7.63 (t, J = 7.6 Hz, 1H), 7.49 (t, J = 7.6 Hz, 2H), 5.55 (s, 1H), 5.27 (s, 1H), 4.53 (dd, J_1 = 15.6 Hz, J_2 = 38.8 Hz, 2H), 4.20 (dd, J_1 = 11.2 Hz, J_2 = 14.4 Hz, 2H), 3.92 (d, J = 10.4 Hz, 2H), 3.49 (d, J = 10.4 Hz, 1H), 3.33 (d, J = 9.6 Hz, 1H), 2.27 (d, J = 10.2 Hz, 1 H), 2.12 (dd, J_1 = 4.4 Hz, J_2 = 12.8 Hz, 1H), 2.02–2.06 (m, 1H), 1.97 (dd, J_1 = 3.2 Hz, J_2 = 8.8 Hz, 2H), 1.69–1.85 (m, 4H), 1.65 (t, J = 8.8 Hz, 1H), 1.45–1.57 (m, 3H), 1.31–1.38 (m, 3H), 1.29 (s, 1H), 1.26 (d, J = 6.8 Hz, 3H), 1.22 (s, 3H), 1.11 (s, 3H), 1.08 (s, 3H), 0.99–1.05 (m, 2H), 0.96 (d, J = 7.0 Hz, 3H), 0.87 (d, J = 6.4 Hz, 3H), 0.75 (s, 3H). HRMS (ESI) m/z calcd for $C_{41}H_{58}O_7$ $[M+H]^+$ 663.4262, found 663.4338.

*4.1.8. (2R,4aR,6aR,6bS,8aS,11R,12S,12aS,14bR,16R,16aR)-2-Ethoxy-2-oxoethyl2-ethyl-16-hydroxy-4a,6a,6b,11,12,14b-hexamethyl-4a,4b,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b,15,16,16a-icosahydro-4H-piceno[3,4-d][1,3]dioxine-8a-carboxylate (**5c**)*

Compound **5c** was prepared similarly according to the procedure of **5a**. θ_{mp} 61–63 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.26 (t, J = 3.2 Hz, 1H), 1.57 (d, J = 15.6 Hz, 1H), 1.52 (t, J = 4.8 Hz, 1H), 1.47 (d, J = 16.0 Hz, 1H), 1.19 (q, J = 7.2 Hz, 2H), 3.85 (td, J_1 = 4.4 Hz, J_2 = 10.0 Hz, 1H), 3.75 (d, J = 10.4 Hz, 1H), 3.25 (d, J = 10.4 Hz, 1H), 3.06 (d, J = 9.6 Hz, 1 H), 2.26 (d, J = 10.8 Hz, 2H), 2.00–2.10 (m, 2H), 1.95 (dd, J_1 = 3.2 Hz, J_2 = 8.8 Hz, 2H), 1.75–1.82 (m, 3H), 1.63 (t, J = 8.8 Hz, 1H), 1.57 (s, 3H), 1.30–1.52 (m, 6H), 1.26 (t, J = 7.2 Hz, 3H), 1.14–1.20 (m, 1H), 1.09 (s, 3H), 1.08 (s, 3H), 1.04 (s, 3H), 0.98 (s, 1H), 0.96 (d, J = 4.0 Hz, 3H), 0.94 (d, J = 2.4 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H), 0.72 (s, 3H). HRMS (ESI) m/z calcd for $C_{37}H_{58}O_7$ $[M+H]^+$ 615.4265, found 615.4233.

*4.1.9. (2R,4aR,6aR,6bS,8aS,11R,12S,12aS,14bR,16R,16aR)-2-Ethoxy-2-oxoethyl16-hydroxy-2-(4-hydroxyphenyl)-4a,6a,6b,11,12,14b-hexamethyl-4a,4b,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b,15,16,16a-icosahydro-4H-piceno[3,4-d][1,3]dioxine-8a-carboxylate (**5d**)*

Compound **5d** was prepared similarly according to the procedure of **5a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 (d, J = 15.6 Hz, 1H), 7.07 (d, J = 8.0 Hz, 1H), 7.01 (s, 1H), 6.83 (dd, J_1 = 2.0 Hz, J_2 = 7.2 Hz, 1H), 5.50 (s, 1H), 5.27 (s, 1H), 4.53 (dd, J_1 = 16.0 Hz, J_2 = 38.8 Hz, 2H), 4.20 (q, J = 7.2 Hz, 2H), 3.91 (d, J = 10.4 Hz, 1H), 3.47 (d, J = 10.0 Hz, 1H), 3.30 (d, J = 9.6 Hz, 1H), 2.27 (d, J = 11.2 Hz, 1H), 2.11 (dd, J_1 = 4.8 Hz, J_2 = 12.4 Hz, 1H), 2.04 (dd, J_1 = 7.0 Hz, J_2 = 12.4 Hz, 1H), 1.97 (dd, J_1 = 4.0 Hz, J_2 = 8.8 Hz, 2H), 1.69–1.84 (m, 1H), 1.65 (t, J = 8.8 Hz, 3H), 1.41–1.55 (m, 1H), 1.31–1.37 (m, 3H), 1.27 (t, J = 7.2 Hz, 3H), 1.20 (s, 3H), 1.11 (s, 3H), 1.07 (s, 3H), 1.01–1.04 (m, 2H), 0.95 (d, J = 7.0 Hz, 3H), 0.91–0.93 (m, 1H), 0.87 (d, J = 8.4 Hz, 3H), 0.79–0.84 (m, 1H), 0.74 (s, 3H). HRMS (ESI) m/z calcd for $C_{41}H_{58}O_8$ $[M+H]^+$ 679.4211, found 679.4214.

*4.1.10. (2R,4aR,6aR,6bS,8aS,11R,12S,12aS,14bR,16R,16aR)-2-Ethoxy-2-oxoethyl16-hydroxy-2-(4-methoxyphenyl)-4a,6a,6b,11,12,14b-hexamethyl-4a,4b,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b,15,16,16a-icosahydro-4H-piceno[3,4-d][1,3]dioxine-8a-carboxylate (**5e**)*

Compound **5e** was prepared similarly according to the procedure of **5a**. θ_{mp} 55–57 °C. HRMS (ESI) m/z calcd for $C_{42}H_{60}O_8$ $[M+H]^+$ 693.4367, found 693.4353.

*4.1.11. (2R,4aR,6aR,6bS,8aS,11R,12S,12aS,14bR,16R,16aR)-2-Ethoxy-2-oxoethyl16-hydroxy-4a,6a,6b,11,12,14b-hexamethyl-2-(4-nitrophenyl)-4a,4b,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b,15,16,16a-icosahydro-4H-piceno[3,4-d][1,3]dioxine-8a-carboxylate (**5f**)*

Compound **5f** was prepared similarly according to the procedure of **5a**. θ_{mp} 63–65 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, J = 8.4 Hz, 2H), 7.70 (d, J = 8.8 Hz, 2H), 5.63 (s, 1H), 5.27 (s, 1H), 4.58 (d, J = 15.6 Hz, 1H), 4.48 (d, J = 15.6 Hz, 1H), 4.20 (q, J = 7.2 Hz, 2H), 3.94 (d, J = 10.4 Hz, 2H), 3.51 (d, J = 10.4 Hz, 1H), 3.34 (d, J = 0.96 Hz, 1H), 2.27 (d, J = 11.2 Hz, 1H), 2.13 (dd, J_1 = 4.8 Hz, J_2 = 12.8 Hz, 1H), 2.02–2.10 (m, 1H), 1.97 (dd, J_1 = 3.2 Hz, J_2 = 8.8 Hz, 2H), 1.70–1.85 (m, 4H), 1.65 (t, J = 8.8 Hz, 1H), 1.43–1.55 (m, 6H), 1.26 (t, J = 7.2 Hz, 3H), 1.20–1.23 (m, 1H), 1.19 (s, 3H), 1.11 (s, 3H), 1.08 (s, 3H), 0.95 (d, J = 6.4 Hz, 3H), 0.87 (d, J = 6.8 Hz, 3H), 0.77–0.91 (m, 4H), 0.75 (s, 3H). HRMS (ESI) m/z calcd for $C_{41}H_{57}NO_9$ $[M+H]^+$ 708.4033, found 708.4030.

4.2. Anti-fibrotic activity

4.2.1. Materials

Asiatic acid (white powder) and SD rats (both males and females, weight: 150–190 g) were obtained from the Experimental Animal Center of Sichuan University. CMC (500 g; catalogue number: 20120320) and CCl_4 (500 mL, catalogue number: 20110701) were obtained from Chengdu Kelong Chemical Reagent Factory. Carp brand rapeseed oil (900 mL) was obtained from Yihai Whole Foods Marketing Co., Ltd. To prepare the CMC solution, 5.0 g of CMC was dissolved in 1 L of pure water. Aspartate aminotransferase matrix solution: $K_2HPO_4{\cdot}3H_2O$ 100 mmol/L, KH_2PO_4 100 mmol/L DL-aspartate 200 mmol/L, α-ketoglutarate 2.0 mmol/L, NaN_3 0.1%; 2.4-dinitrophenylhydrazine solution: concentrated HCl 100 mmol/L, 2.4-dinitrophenyl hydrazine 1.0 mmol/L; NaOH 4.0 mol/L.

4.2.2. Methods

AST measurement method: Rate method (aspartate aminotransferase enzyme assay kit): Aspartate amino transferase in serum or plasma to alpha ketoglutaric acid and aspartic acid, transfer amino and keto, glutamic acid and oxaloacetic acid generation, oxaloacetate decarboxylation to pyruvate in the reaction process. Pyruvate and 2.4- two dinitrophenylhydrazine reaction: 2.4- two nitrohydrazone, red brown color in alkaline solution. Colorimetric, by checking the standard curve, one can obtain enzyme activity.

4.2.3. Establishment of anti-fibrosis model

We have synthesized a series of asiatic acid derivatives to study the effect of these structural modifications in a rodent CCl_4-induced liver injury model. In this model, CCl_4 initially activates liver microsomal cyctochrome P450 to release free radicals. These free radicals then attack the phospholipid molecules present in the membranes of liver cells, which compromises the structural integrity of the membrane and ultimately leads to the release of intracellular enzymes such as AST and ALT into the blood stream. We observed significantly increased activity of AST and ALT in serum, characteristic of a successful CCl_4-induced liver injury model.

4.2.4. Biochemical parameters of liver function

The activities of aspartate transaminase (AST) and alanine transaminase (ALT) in serum were measured by using kits [School of Pharmacy, Sichuan University, China].

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