Modification of C11, C28, C2,3,23 or C2,23,28 Functional Groups on Asiatic Acid and Evaluation of Hepatoprotective Effects

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For the development of novel hepatoprotective agents, C11, C28, C2,3,23 or C2,23,28 functional groups on asiatic acid were modified, and their hepatoprotective effects were evaluated. Most of the prepared compounds displayed potent hepatoprotective activities against CCl_4- and galactosamine (GalN)-induced hepatotoxicity. Especially, compounds **16** and **20** showed the most significant hepatoprotective effects against GalN-induced hepatotoxicity (54.2% and 46.4% protection at 50 mM, respectively).

Key Words : Asiatic acid, Hepatoprotective effect, Structural modification, Hepatotoxicity

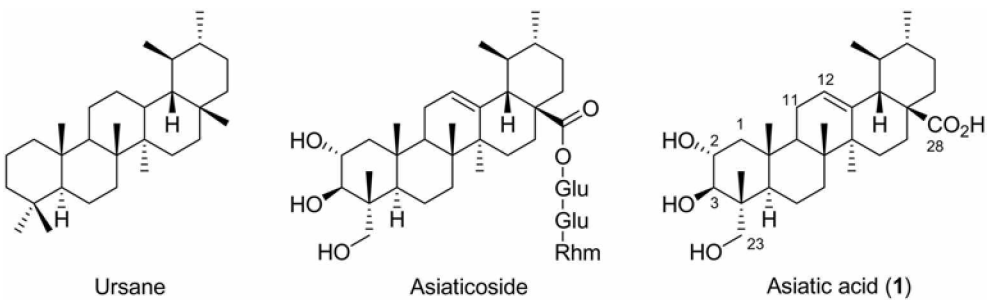
Introduction

Liver is one of the most important organs in the human body performing excretion and metabolism. Acute and chronic hepatic disease has been recently increased but efficient drugs without side effects have not been developed. It has been reported that ursolic and oleanoic acid which have ursane structure showed strong hepatoprotective activity.[1-6] Asiatic acid, whose structure is derived from ursane skeleton, is one of the triterpenoids isolated from *Centella Asiatica*,[7] and has moderate hepatoprotective activity.[8] Asiatic acid can be easily prepared from hydrolysis of asiaticoside. Modification of functional groups of asiatic acid may provide valuable information of structure-activity relationships for the development of novel hepatoprotective agents. Previously, we reported modifications of C2 and C2,3,23,28 functional group on asiatic acid and evaluation of their hepatoprotective effects.[9] In connection with these studies, we modified C11, C28, C2,3,23 or C2,3,28 functional groups on asiatic acid and evaluated their hepatoprotective effects.

Experimental

Material and methods. All reagents were purchased from Aldrich Chemical (www.sigma-aldrich.com) and used without further purification. Unless otherwise indicated, anhydrous solvent were distilled over CaH_2 or sodium benzophenone ketyl prior to use. Thin-layer chromatography (TLC) and column chromatography were performed with Kieselgel 60 F_{254} (Merck) and silica gel Kieselgel 60, (230-400 mesh, Merck), respectively. Compounds containing aromatic ring were visualized on TLC plates with UV light, and compounds containing oxygen were visualized on TLC plates with *p*-anisaldehyde staining solution. Nuclear magnetic resonance (NMR) spectra were taken on a Bruker AMX 250 MHz for ^{1}H NMR and 62.5 MHz for ^{13}C NMR, and tetramethylsilane (TMS) was used as an internal standard. Chemical shifts (δ) were recorded in ppm, and coupling constants (J) in Hz. Melting points were determined in open capillary tubes on electrothermal 1A 9100 digital melting point apparatus and were uncorrected.

2α,3β,23-Triacetoxyurs-12-ene-28-oic acid (2). To a stirred solution of **1** (3.00 g, 6.14 mmol) in pyridine (60 mL) was added acetic anhydride (5.80 mL, 61.40 mmol). The mixture was stirred at 20 °C for 8 h. After dilution with ethyl acetate (250 mL), the mixture was washed with aqueous 1 M HCl (50 mL × 5) and saturated $CuSO_4$ (25 mL × 2) and saturated NaCl solution (50 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which



Ursane Asiaticoside Asiatic acid (**1**)

Figure 1. Structures of ursane, asiaticoside and asiatic acid.

was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:3, v:v) to yield a white solid (2.2 g, 58.3%).

TLC (EtOAc:*n*-hexane = 1:2, v:v), R_f = 0.25. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.24 (br, 1H), 5.15-5.09 (m, 1H), 5.08 (d, J = 10.3 Hz, 1H), 3.82 (d, J = 11.7 Hz, 1H), 3.58 (d, J = 11.7 Hz, 1H), 2.19 (d, J = 11.1 Hz, 1H), 2.09, 2.03, 1.98, 1.10, 1.07, 0.88, 0.76 (s, each 3H), 0.94 (d, J = 5.6 Hz, 3H), 0.85 (d, J = 6.4 Hz, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C12, C13, C28 carbon only) δ 178.65, 138.34, 125.67.

2α,3β,23-Triacetoxyurs-11-oxo-12-ene-28-oic acid (3). A solution of **2** (1.73 g, 2.81 mmol) and $Na_2Cr_2O_7{\cdot}2H_2O$ (2.10 g, 7.04 mmol) in 100 mL of acetic acid was refluxed for 5 h. The mixture was cooled to 20 °C and neutralized with 10% $NaHCO_3$ solution to pH 7-8, diluted with ethyl acetate (150 mL) and washed with water (50 mL × 5), and saturated NaCl solution (50 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of CH_2Cl_2/MeOH (30:1, v:v) to yield a light yellow solid (1.64 g, 92.7%).

TLC (CH_2Cl_2:MeOH = 9:1, v:v), R_f = 0.55. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.62 (s, 1H), 5.29 (dt, J = 11, 4.5 Hz, 1H), 5.05 (d, J = 10.3 Hz, 1H), 3.82 (d, J = 11.9 Hz, 1H), 3.61 (d, J = 11.9 Hz, 1H), 3.2 (dd, J = 12.8, 4.6 Hz, 1H), 2.09, 2.02, 1.96, 1.30, 1.29, 0.90, 0.89 (s, each 3H), 0.97 (d, J = 6.0 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H). ^{13}C NMR (62.5 MHz, pyridine-d_5, C11, C12, C13, C28 carbon only) δ 199.67, 179.23, 164.54, 131.34.

2α,3β,23-Trihydroxyurs-11-oxo-12-ene-28-oic acid (4). To a stirred solution of **3** (0.93 g, 1.48 mmol) in methanol (100 mL) and water (20 mL) was added potassium carbonate (1.23 g, 8.88 mmol) and stirred for 6 h at 20 °C. The mixture was neutralized with aqueous 1 M HCl to pH 2-3. The solvent was evaporated under reduced pressure to give a light yellow solid, which was purified by silica gel chromatography with a gradient elution of CH_2Cl_2/MeOH (9:1, v:v) to yield a white solid (0.60 g, 80.6%).

TLC (CH_2Cl_2:MeOH = 9:1, v:v), R_f = 0.21. ^{1}H NMR (250 MHz, pyridine-d_5) δ 6.0 (s, 3H), 4.38 (m, 1H), 4.25 (d, J = 9.7 Hz, 2H), 3.73 (dd, J = 8.5, 4.0 Hz, 1H), 3.7 (d, J = 10.5 Hz, 1H), 2.75 (s, 1H), 2.66 (d, J = 11.3 Hz, 1H), 1.46, 1.28, 1.22, 1.06, 0.85 (s, each 3H), 0.80 (d, J = 6.4 Hz, 3H). ^{13}C NMR (62.5 MHz, pyridine-d_5, C11, C12, C13, C28 carbon only) δ 199.81, 179.58, 164.24, 131.07.

2α-Benzyloxy-3β,23-isopropylidenedioxy-28-hydroxyurs-12-ene (6). To the solution of **5** (1.88 g, 2.97 mmol)[9] in dry tetrahydrofuran (20 mL) was added lithium aluminum hydride (1.0 M solution in tetrahydrofuran, 3.0 mL) and refluxed for 1 h. The mixture was filtered and evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (100 mL) and washed with water (60 mL × 3) and saturated NaCl solution (50 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:4, v:v) to yield a white solid (1.5 g, 83.5%).

TLC (EtOAc:*n*-hexane = 1:5, v:v), R_f = 0.15. ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.35-7.25 (m, 5H), 5.14 (br, 1H), 4.82, 4.59 (AB quartet, J = 11.7 Hz, 2H), 3.64-3.46 (m, 5H), 3.19 (d, J = 10.9 Hz, 1H), 2.09 (d, 1H), 1.48, 1.47 (s, each 3H), 1.10, 1.10, 1.03, 0.96, 0.93 (s, each 3H). ^{13}C NMR (62.5 MHz, pyridine-d_5, C12, C13, C28 carbon only) δ 138.87, 124.31, 69.73.

2α-Benzyloxy-3β,23,28-trihydroxyurs-12-ene (7). To the solution of **6** (1.30 g, 2.15 mmol) in THF (50 mL) was added aqueous 1 M HCl (8 mL) at 20 °C and stirred for 10 h. The mixture was evaporated under reduced pressure to remove THF. The residue was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:2, v:v) to yield a white solid (1.1 g, 90.6%).

TLC (EtOAc:*n*-hexane = 1:2, v:v), R_f = 0.15. ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.38-7.28 (m, 5H), 5.15 (br, 1H), 4.68, 4.45 (AB quartet, J = 11.2 Hz, 2H), 3.64 (d, J = 10.5 Hz, 1H), 3.59-3.50 (m, 3H), 3.40 (d, J = 10.6 Hz, 1H), 3.19 (d, J = 11.2 Hz, 1H), 2.18 (d, 1H), 1.10, 1.05, 0.99, 0.93, 0.89 (s, each 3H). ^{13}C NMR (62.5 MHz, pyridine-d_5, C12, C13, C28 carbon only) δ 139.68, 123.81, 69.55.

2α,3β,23,28-Tetrahydroxyurs-12-ene (8). **7** (0.87 g, 1.54 mmol) was dissolved in absolute MeOH (20 mL), and 10% Pd/C (100 mg) was added to the solution under argon atmosphere. The mixture was hydrogenolyzed for 3 h under 60 psi. The mixture was filtered using Celite pad to remove Pd/C and evaporated under reduced pressure. The residue was purified by silica gel chromatography with a gradient elution of CH_2Cl_2/MeOH (20:1, v:v) to yield a white solid (0.65 g, 88.9%).

TLC (CH_2Cl_2:MeOH:AcOH = 9:1:0.1, v:v:v), R_f = 0.41. ^{1}H NMR (250 MHz, pyridine-d_5) δ 5.19 (br, 1H), 4.3-4.15 (m, 3H), 3.89 (d, J = 10.5 Hz, 1H), 3.72 (d, J = 10.5 Hz, 1H), 3.59 (s, 1H), 3.45 (d, J = 10.5 Hz, 1H), 1.12, 1.10, 1.08, 1.03, 0.91 (s, each 3H), 0.90 (d, J = 6.4 Hz, 3H). ^{13}C NMR (62.5 MHz, pyridine-d_5, C12, C13, C28 carbon only) δ 138.99, 124.28, 69.87.

3β,23-Diacetoxyurs-2-oxo-12-ene-28-oic acid (10). To a stirred solution of **9** (150 mg, 0.31 mmol)[9] in THF (5 mL) was added 4-dimethylaminopyridine (10 mg). After stirring for 30 min at 20 °C, acetic anhydride (0.17 mL, 1.85 mmol) was added, and the mixture was stirred for 2 h at 20 °C. The mixture was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (50 mL) and washed with water (20 mL × 2) and saturated NaCl solution (20 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:2, v:v) to yield a white solid (130 mg, 73.5%).

TLC (EtOAc:*n*-hexane = 1:2, v:v), R_f = 0.22. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.25 (br, 1H), 5.23 (s, 1H), 4.05 (d, J = 11.7 Hz, 1H), 3.74 (d, J = 11.8 Hz, 1H), 2.47 (d, J = 12.3 Hz, 1H), 2.17, 2.11, 1.12, 0.96, 0.80, 0.77 (s, each 3H), 0.93 (d, J = 6.0 Hz, 3H), 0.86 (d, J = 6.3 Hz, 3H). ^{13}C NMR (62.5

MHz, $CDCl_3$, C2, C3, C12, C13, C23, C28 carbon only) δ 204.41, 183.84, 171.01, 170.62, 138.55, 125.32.

3β-Hydroxy-23-acetoxyurs-2-oxo-12-ene-28-oic acid (11). To a stirred solution of **9** (200 mg, 0.41 mmol) in THF (10 mL) was added acetyl chloride (0.088 mL, 1.23 mmol) at 0 °C. After stirring for 5 min, triethylamine (0.34 mL, 2.46 mmol) was added at 0 °C, and the mixture was stirred for 12 h at 20 °C. The mixture was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (50 mL) and washed with water (20 mL × 2) and saturated NaCl solution (20 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:1, v:v) to yield a white solid (90 mg, 41.5%).

TLC (EtOAc:*n*-hexane = 1:1, v:v), R_f = 0.21. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.25 (br, 1H), 4.27 (s, 1H), 4.17 (d, J = 11.5 Hz, 1H), 3.86 (d, J = 11.5 Hz, 1H), 2.53 (d, J = 12.3 Hz, 1H), 2.11, 1.12, 0.96, 0.80, 0.77 (s, each 3H), 0.93 (d, J = 6.0 Hz, 3H), 0.86 (d, J = 6.3 Hz, 3H), ^{13}C NMR (62.5 MHz, $CDCl_3$, C2, C12, C13, C23, C28 carbon only) δ 211.31, 183.75, 171.01, 138.63, 125.26.

3β,23-Diacetoxyurs-12-ene-28-oic acid (13). To a stirred solution of **12** (150 mg, 0.32 mmol)[9] in THF (5 mL) was added 4-dimethylaminopyridine (10 mg). After stirring for 30 min, acetic anhydride (0.3 mL, 3.17 mmol) was added, and the mixture was stirred for 2 h at 20 °C. The mixture was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (50 mL) and washed with water (20 mL × 2) and saturated NaCl solution (20 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:4, v:v) to yield a white solid (140 mg, 78.6%).

TLC (EtOAc:*n*-hexane = 1:2, v:v), R_f = 0.35. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.24 (br, 1H), 4.79 (m, 1H), 3.88 (d, J = 11.6 Hz, 1H), 3.69 (d, J = 11.6 Hz, 1H), 2.19 (d, J = 11.2 Hz, 1H), 2.07, 2.03, 1.07, 0.99, 0.82, 0.77 (s, each 3H), 0.94 (d, J = 6.5 Hz, 3H), 0.86 (d, J = 6.3 Hz, 3H), ^{13}C NMR (62.5 MHz, $CDCl_3$, C3, C12, C13, C23, C28 carbon only) δ 183.97, 171.44, 171.12, 138.35, 126.02.

3β-Hydroxy-23-trimethylacetoxyurs-12-ene-28-oic acid (14). To a stirred solution of **12** (150 mg, 0.32 mmol) in THF (5 mL) was added 4-dimethylaminopyridine (10 mg). After stirring for 30 min, trimethylacetic anhydride (0.64 mL, 3.17 mmol) was added, and the mixture was stirred for 2 h at 20 °C. The mixture was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (50 mL) and washed with water (20 mL × 2) and saturated NaCl solution (20 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:2, v:v) to yield a white solid (120 mg, 67.3%).

TLC (EtOAc:*n*-hexane = 1:2, v:v). R_f = 0.20. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.25 (br, 1H), 4.18 (d, J = 11.5 Hz, 1H), 3.81 (d, J = 11.4 Hz, 1H), 3.39 (m, 1H), 2.19 (d, J = 11.1 Hz, 1H), 1.23 (s, 3H), 1.06, 0.97, 0.78, 0.76 (s, each 3H), 0.94 (d, J = 7.2 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C12, C13, C23, C28 carbon only) δ 183.78, 179.16, 138.26, 126.19.

Methyl 2α,23-diacetoxy-3β-hydroxyurs-12-ene-28-oate (16). To a stirred solution of **15** (0.50 g, 1.00 mmol)[9] and acetyl chloride (1.06 mL, 14.90 mmol) in THF (15 mL) was added triethylamine (1.5 mL, 14.90 mmol) at 0 °C. The mixture was stirred for 3 h at 20 °C. The mixture was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (60 mL) and washed with water (30 mL × 3) and saturated NaCl solution (30 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:2, v:v) to yield a white solid (230 mg, 39.2%).

TLC (EtOAc:*n*-hexane = 1:2, v:v). R_f = 0.21. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.26 (t, J = 3.4 Hz, 1H), 5.02 (m, 1H), 4.08 (d, J = 11.5 Hz, 1H), 3.94 (d, J = 11.4 Hz, 1H), 3.61 (s, 3H), 3.50 (d, J = 10.1 Hz, 1H), 2.23 (d, J = 11.3 Hz, 1H), 2.08, 2.07 (s, each 3H), 1.07, 1.07, 0.85, 0.75 (s, each 3H), 0.94 (d, J = 5 Hz, 3H), 0.84 (d, J = 6.2 Hz, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C2, C12, C13, C23, C28 carbon only) δ 178.40, 171.86, 171.40, 138.68, 125.12.

Methyl 3β,23-isopropylidenedioxyurs-2-oxo-12-ene-28-oate (18). A solution of pyridinium dichromate (56 mg) and acetic anhydride (0.05 mL) in dry CH_2Cl_2 (10 mL) was stirred 30 min at 20 °C. To the reaction solution was added **17** (100 mg, 0.18 mmol)[8] in dry CH_2Cl_2 (5 mL) slowly and refluxed for 2 h. The mixture was diluted with ethyl acetate (80 mL) and filtered to remove precipitate and washed with water (30 mL × 3) and saturated NaCl solution (30 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:4, v:v) to yield a white solid (71.8 mg, 73.8%).

TLC (EtOAc:*n*-hexane = 1:4, v:v). R_f = 0.22. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.25 (br, 1H), 4.40 (s, 1H), 3.60 (s, 3H), 3.70, 3.59 (AB quartet, J = 10.4 Hz, 2H), 2.25 (d, J = 10.8 Hz, 1H), 2.39, 2.13 (AB quartet, J = 12.4 Hz, 2H), 0.95 (d, J = 5.6 Hz, 3H), 0.86 (d, J = 6.0 Hz, 3H), 1.52, 1.45, 1.14, 1.05, 1.01, 0.74 (s, each 3H), ^{13}C NMR (62.5 MHz, $CDCl_3$, C2, C12, C13, C28 carbon only) δ 211.59, 179.11, 138.87, 125.08.

Methyl 3β,23-dihydroxyurs-2-oxo-12-ene-28-oate (19). To the solution of **18** (30 mg, 0.055 mmol) in THF (0.16 mL) was added aqueous 1 M HCl (5 mL) and stirred for 2 h at 20 °C. The mixture was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (20 mL), washed with water (5 mL × 2) and saturated NaCl solution (10 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at

reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:2, v:v) to yield a white solid (27.8 mg, 89.3%).

TLC (EtOAc:*n*-hexane = 1:2, v:v), R_f = 0.20. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.25 (br, 1H), 4.36 (s, 1H), 3.61 (s, 3H), 3.52 (s, 2H), 2.50 (d, J = 12.2 Hz, 1H), 2.25 (d, J = 11.2 Hz, 1H), 2.12 (d, J = 12.3 Hz, 1H), 1.13, 0.92, 0.75, 0.60 (s, each 3H), 0.95 (d, J = 5.6 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$, C2, C12, C13, C28 carbon only) δ 211.65, 177.98, 138.66, 125.01.

Methyl 3β-hydroxy-23-acetoxyurs-2-oxo-12-ene-28-oate (20). To a stirred solution of **19** (300 mg, 0.60 mmol) in CH_2Cl_2 (5 mL) was added acetyl chloride (0.128 mL, 1.80 mmol) at 0 °C. After stirring for 5 min, triethylamine (0.5 mL, 3.60 mmol) was added at 0 °C, and the mixture was stirred for 2 h at 20 °C. The mixture was evaporated under reduced pressure to remove THF. The residue was diluted with ethyl acetate (50 mL) and washed with water (20 mL × 2) and saturated NaCl solution (30 mL), the organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:4, v:v) to yield a white solid (158 mg, 48.5%).

TLC (EtOAc:*n*-hexane = 1:4, v:v), R_f = 0.21. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.26 (t, J = 3.4 Hz, 1H), 4.26(s, 1H), 4.18 (d, J = 11.5 Hz, 1H), 3.86 (d, J = 11.5 Hz, 1H), 3.60 (s, 3H), 2.53 (d, J = 12.3 Hz, 1H), 2.26 (d, J = 11.2 Hz, 1H), 2.14 (d, J = 12.3 Hz, 1H), 2.11 (s, 3H), 1.13, 0.92, 0.75, 0.66 (s, each 3H), 0.95 (d, J = 5.6 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H), ^{13}C NMR (62.5 MHz, $CDCl_3$, C2, C12, C13, C23, C28 carbon only) δ 211.28, 178.31, 170.97, 138.90, 125.03.

Methyl 3β-hydroxy-23-propionyloxyurs-2-oxo-12-ene-28-oate (21). To a stirred solution of **19** (200 mg, 0.40 mmol) in CH_2Cl_2 (5 mL) was added propionyl chloride (0.104 mL, 1.20 mmol) at 0 °C. After stirring for 5 min, triethylamine (0.33 mL, 2.40 mmol) was added at 0 °C, and the mixture was stirred for 2 h at 20 °C. The mixture was evaporated under reduced pressure to remove CH_2Cl_2. The residue was diluted with ethyl acetate (50 mL) and washed with water (20 mL × 2) and saturated NaCl solution (30 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:4, v:v) to yield a white solid (54.5 mg, 22.3%).

TLC (EtOAc:*n*-hexane = 1:4, v:v), R_f = 0.20. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.26 (t, J = 3.4 Hz, 1H), 4.27 (s, 1H), 4.21 (d, J = 11.5 Hz, 1H), 3.86 (d, J = 11.5 Hz, 1H), 3.61 (s, 3H), 2.53 (d, J = 12.3 Hz, 1H), 2.40 (q, J = 7.6, 2H), 2.26 (d, J = 10.9 Hz, 1H), 2.13 (d, J = 12.7 Hz, 1H), 1.19 (t, J = 7.5 Hz, 3H), 1.12, 0.92, 0.75, 0.67 (s, each 3H), 0.95 (d, J = 5.6 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H), ^{13}C NMR (62.5 MHz, $CDCl_3$, C2, C12, C13, C23, C28 carbon only) δ 211.31, 178.32, 174.23, 138.89, 125.06.

Methyl 3β-hydroxy-23-trimethylacetoxyurs-2-oxo-12-ene-28-oate (22). To a stirred solution of **19** (200 mg, 0.40 mmol) in CH_2Cl_2 (5 mL) was added pivaloyl chloride (0.148 mL, 1.20 mmol) at 0 °C. After stirring for 5 min, triethylamine (0.33 mL, 2.4 mmol) was added at 0 °C, and the mixture was stirred for 2 h at 20 °C. The mixture was evaporated under reduced pressure to remove CH_2Cl_2. The residue was diluted with ethyl acetate (50 mL) and washed with water (20 mL × 2) and saturated NaCl solution (30 mL). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave a light yellow solid, which was purified by silica gel chromatography with a gradient elution of EtOAc/*n*-hexane (1:5, v:v) to yield a white solid (112.9 mg, 48.3%).

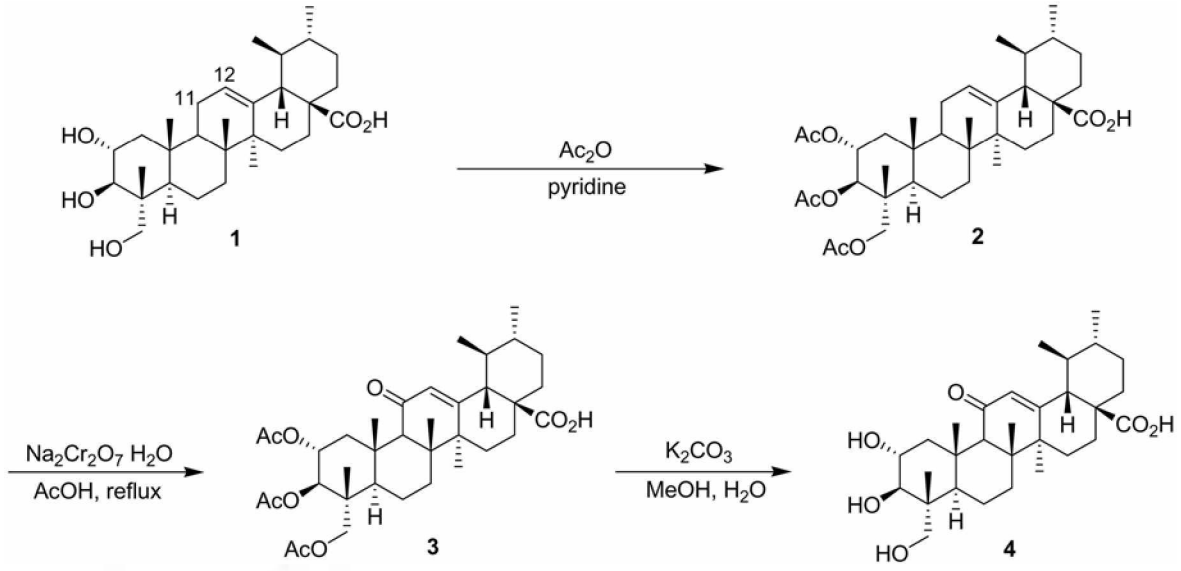
TLC (EtOAc:*n*-hexane = 1:5, v:v), R_f = 0.21. ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.26 (t, J = 3.4 Hz, 1H), 4.27 (s, 1H), 4.20 (d, J = 11.5 Hz, 1H), 3.83 (d, J = 11.5 Hz, 1H), 3.61 (s, 3H), 2.54 (d, J = 12.2 Hz, 1H), 2.26 (d, J = 11.1 Hz, 1H), 2.10 (d, J = 12.4 Hz, 1H), 1.26 (s, 9H), 1.10, 0.92, 0.75, 0.67 (s, each 3H), 0.94 (d, J = 6.0 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H), ^{13}C NMR (62.5 MHz, $CDCl_3$, C2, C12, C13, C23, C28 carbon only) δ 211.30, 178.33, 178.08, 138.84, 125.09.

Primary cell cultured rat hepatocytes assay. Rat hepatocytes were prepared from male Wistar rats by a collagenase perfusion technique of Berry and Friend with minor modification.[10] 24 h after the isolated rat hepatocytes were plated, the cultured cells were exposed to a culture containing 5 mM of chloroform or glucosamine either with, or without the prepared compounds along with asiatic acid and silymarin. After 1.5 h, the activities of glutamic pyruvic transaminase (GPT) released into the culture medium were determined by the method of Reitman-Frankel.[11] All data are expressed as the mean ±SD. The evaluation of statistical significance was determined by "the one-way ANOVA" using a computerized statistical package. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

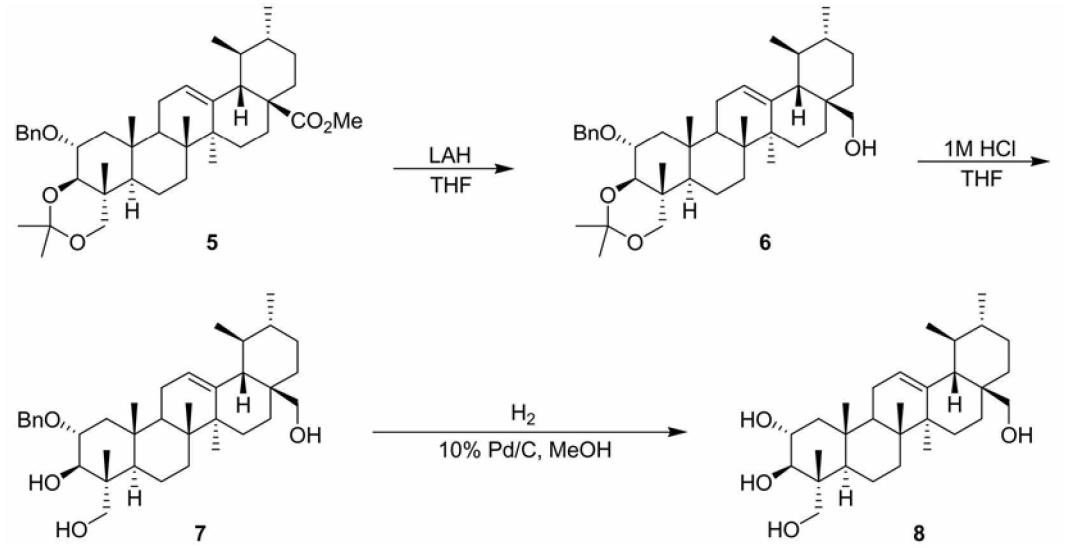
Results and Discussion

Chemistry. For the preparation of the asiatic acid derivative whose methylene group at C11 position was modified to keto group, allylic oxidation was performed. Because olefinic carbon at C12 position is known to be very resistant to epoxidation, catalytic hydrogenation, and dihydroxylation, we investigated to modify this position by allylic oxidation, since structurally similar compounds has been reported to be modified by allylic oxidation.[12] Acetylation of asiatic acid (**1**) with acetic anhydride gave protected intermediate **2** in 58.3% yield, which was converted to α,β-unsaturated ketone **3** in 92.7% yield by the oxidation with $Na_2Cr_2O_7$ in the presence of acetic acid.[13] The final product, trihydroxy compound **4** was obtained in 80.6% yield by deacetylation of **3** (Scheme 1).

For the modification of C28-carboxylic acid moiety at C28 position (Scheme 2), intermediate **5**[8] was reduced with lithium aluminum hydride ($LiAlH_4$) to give hydroxymethyl intermediate **6** in 83.5% yield. Deprotection of acetonide group of **6** with aqueous 1 M HCl gave diol intermediate **7** in 90.6% yield, followed by deprotection of benzyl moiety by hydrogenolysis



Scheme 1. Synthesis of C11-keto asiatic acid.



Scheme 2. Synthesis of C28-hydroxymethyl asiatic acid.

with H_2 and 10% Pd/C gave a final product **8** in 88.9% yield, in which carboxylic acid moiety on asiatic acid was converted to hydroxymethyl moiety.

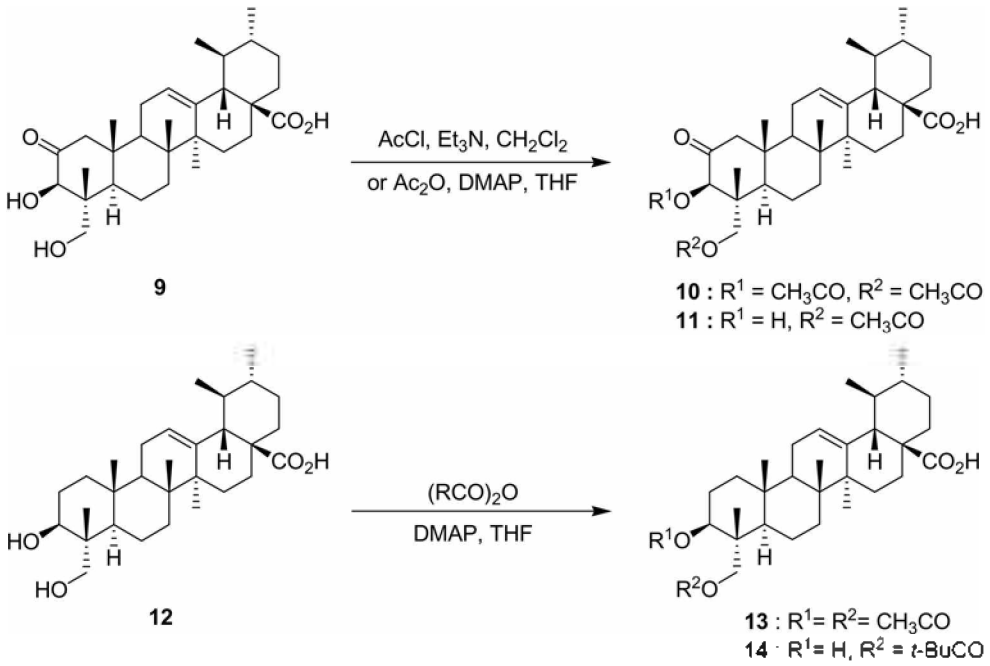
For the preparation of C2-oxo asiatic acid derivatives (**10**, **11**), diol compound **9**[8] was treated with acetyl chloride or acetic anhydride, to form 3β,23-acetoxy compound **10** in 73.5% yield or 23-acetoxy compound **11** in 41.5% yield, respectively (Scheme 3).

For the preparation of C2-deoxygenated asiatic acid (**13**, **14**), intermediate **12**[8] was treated with acetic anhydride or trimethylacetic anhydride in the presence of DMAP, to afford 3β,23-diacetoxy substituted compound **13** in 78.6% yield and 23-trimethylacetoxy substituted compound **14** in 67.3% yield, respectively.

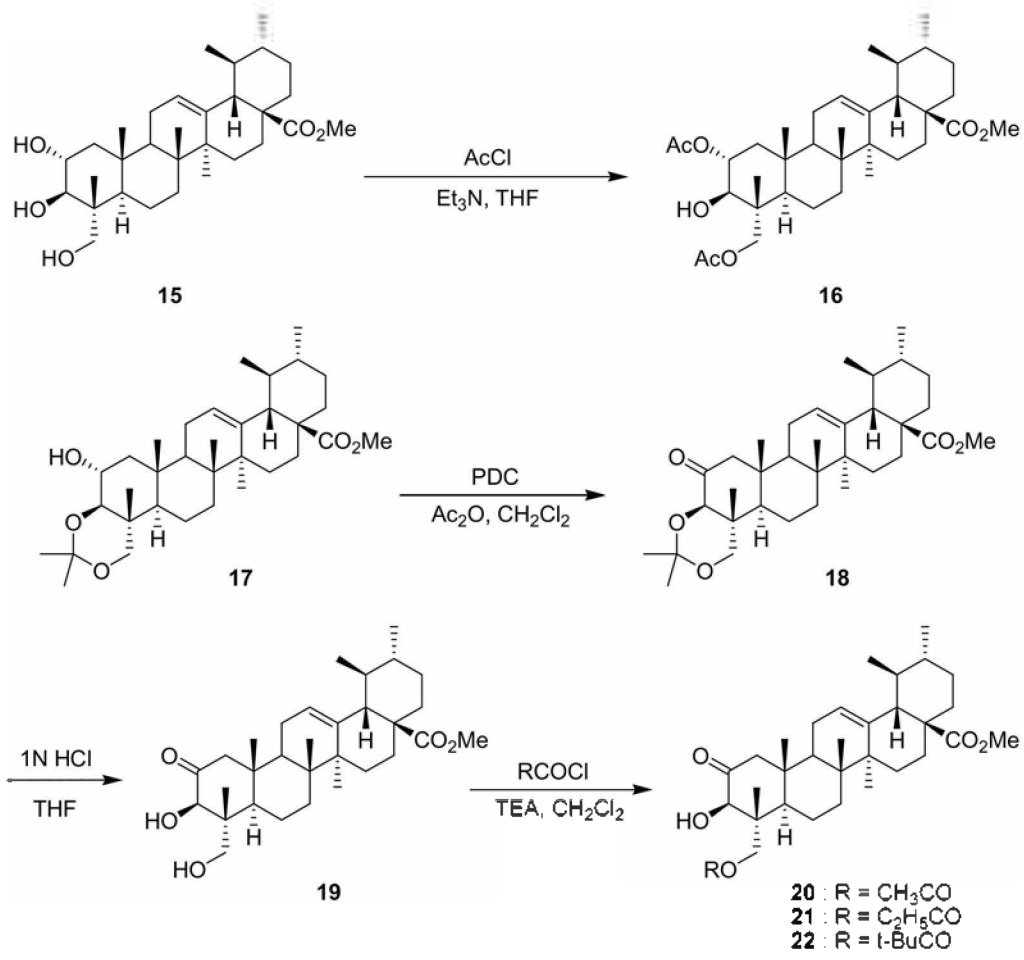
For the preparation of 2α,23-dihydroxy substituted compound **16**, methyl ester **15**[8] was treated with acetyl chloride and triethylamine in THF to provide **16** in 39.2% yield (Scheme 4).

For the preparation of 23-acyl substituted derivatives (**20**-**22**), methyl ester **17**[8] was oxidized with PDC in CH_2Cl_2 under reflux to give 2-oxo compound **18** in 73.8% yield. Deprotection of **18** with aqueous 1 M HCl gave diol compound **19** in 89.3% yield, which was treated with acetyl chloride, propionyl chloride or pivaloyl chloride in CH_2Cl_2, to form **20**, **21** or **22** in 48.5%, 22.3% and 48.3% yield, respectively (Scheme 4).

Pharmacology. For the evaluation of hepatoprotective activities, hepatotoxicity was artificially induced by administration of galactosamine or CCl_4 into primary cultured rat hepatocytes. Prepared compounds were administered with different concentration, and relative glutamic pyruvic transaminase (GPT) activities were observed according to recovery of enzyme activities by the Reitman-Frankel method.[11] Hepatoprotective effect was indicated as % value. (Normal GPT activity: 100%, intoxicated GPT activity: 0%). Hepato-



Scheme 3. Synthesis of C2,3,23-modified asiatic acid.



Scheme 4. Synthesis of C2,23,28-modified asiatic acid.

toxicity induced by CCl_4 was reported to be due to lipid peroxide which was trichloromethyl free radical ($\cdot CCl_3$) metabolite bound with intracellular proteins and lipids by action of cytochrome P-450 dependent mixed oxidase. Hepatotoxicity

Table 1. Hepatoprotective effects of the prepared compounds

Compound	CCl_4-induced	GalN-induced
	Protection (%) at 50 μM	Protection (%) at 50 μM
Asiatic acid (**1**)	1.0	23.1
3	NE	13.0
4	25.6	NE
8	39.7	NE
10	22.0	14.4
11	35.9	26.5
13	NE	22.3
14	NE	37.6
16	NE	54.2
20	NE	46.4
21	NE	21.0
22	NE	18.5
Silymarin	54.7	NE

NE: not effective.

induced by galactosamine has similarities with viral hepatitis in function and formation. Galactosamine was reported to inhibit RNA and protein synthesis, which was due to alteration of amount and metabolite of uracil nucleotides in liver. Galactosamine decreased the biosynthesis of biomacromolecules related to uracil nucleotides, such as UDP-glucuronic acid, which resulted in damage of related cells and cellular organelles.

Hepatoprotective activities of prepared compounds were evaluated. Silymarin was utilized as a reference compound to compare hepatoprotective activities with tested compounds. Silymarin has a very potent hepatoprotective activity (54.7%) against CCl_4-induced hepatotoxicity, but does not show any activity against galactosamine(GalN)-induced hepatotoxicity. Asiatic acid nearly does not have hepatoprotective activity against CCl_4-induced hepatoxicity, but moderate activity against GalN-induced hepatotoxicity. Most of the tested compounds showed considerable hepatoprotective activities (Table 1).

Modification of asiatic acid into keto group at C11 position (compound **4**) and hydroxylmethyl group at C28 position (compound **8**) increased the hepatoprotective effect (25.6% and 39.7%, respectively) against CCl_4-induced hepatotoxicity. Modification of hydroxyl group into keto group at C2 position (compounds **10**, **11**) increased the hepatoprotective effect (22.0% and 35.9%, respectively) against CCl_4-induced hepatotoxicity and maintained hepatoprotective activity against GalN-induced hepatotoxicity. Acylation on C23 carbon (compounds **16**, **20**, **21**, **22**) or deoxygenation on C2 position (compounds **13**, **14**) of asiatic acid completely disappeared hepatoprotective effect against CCl_4-induced hepatotoxicity. Generally, methyl ester derivatives on C28 position showed the similar or higher hepatoprotective activity against GalN-induced hepatotoxicity. Especially, C2,C23-diacetyl methyl ester (compound **16**) and C2-oxo,C23-acetyl methyl ester (compound **20**) derivatives displayed the most potent hepatoprotective activity (54.2% and 46.4%, respectively) against GalN-induced hepatotoxicity.

Conclusions

In conclusion, eleven asiatic acid derivatives by the modification of C11, C28, C2,3,23 or C2,23,28 functional groups were prepared, and their hepatoprotective activity against CCl_4-induced and GalN-induced hepatotoxicity were evaluated. In addition, structure-activity relationship study of the modified compounds was performed. Among the prepared compounds, compound **16** and **20** showed the most significant hepatoprotective activity against GalN-induced hepatotoxicity.

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