Anti-HIV Triterpene Acids from Geum japonicum

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The methanolextract from the whole plant of Geum japonicum was found to inhibit the human immunodeficiency virus (HIV-1) protease. Through bioassay-directed fractionation of the extract, a new triterpene acid along with five known triterpene acids, ursolic acid, epipomolic acid, maslinic acid, euscaphic acid, and tormentic acid, were isolated. The structure of the new compound was determined by spectral means including 1H–1H COSY, HMQC, HMBC, and NOE experiments to be 2R,19R-dihydroxy-3-oxo-12-ursen-28-oic acid (1). Of these compounds, 1, ursolic acid, and maslinic acid showed potent inhibitory activity against HIV-1 protease.

Introduction

The whole plant of Geum japonicum Thunb. (Rosaceae) has been used as a diuretic in traditional Chinese medicine.1 From this plant, several hydrolysable tannins and triterpenoids have been isolated.1-3 In a previous study of G. japonicum aqueous extract we isolated geponin and gallic aldehyde, which showed antiviral activity.4

In the course of a continuing search for potential plant anti-HIV agents,5 the methanol extract of the whole plant of G. japonicum was found to show significant inhibitory activity against HIV-1 protease. In this paper, we describe the isolation and structure elucidation of a new triterpene acid, 2α,19α-dihydroxy-3-oxo-12-ursen-28-oic acid (1), along with five known triterpene acids, ursolic acid (2), epipomolic acid (3), maslinic acid (4), euscaphic acid (5), and tormentic acid (6) (Figure 1), and their inhibitory activity against HIV-1 protease.

Results and Discussion

A methanol extract of the whole plant of G. japonicum was successively partitioned into hexane- and ethyl acetate-soluble fractions. Biological evaluation of the extract and fractions indicated that most of the activity was in the ethyl acetate-soluble fraction. The active EtOAc-soluble fraction was then subjected to silica gel column chromatography to give a new triterpene acid (1) together with five known triterpene acids, ursolic acid (2), epipomolic acid (3), maslinic acid (4), euscaphic acid (5), and tormentic acid (6). The structures of these compounds, 1, ursolic acid, and maslinic acid showed potent inhibitory activity against HIV-1 protease.

Figure 1. Structures of compounds 1-6.
compounds were elucidated on the basis of various spectroscopic means.

Compound (1) was obtained as a colorless amorphous powder. The infrared (IR) spectrum indicated the presence of hydroxyl group (3600–3200 cm⁻¹), carboxylic acid group (1764 cm⁻¹), and double bond (1650 cm⁻¹). Its molecular formula was determined to be C₃₀H₄₆O₅ by high-resolution mass spectrometry (M⁺ 486.3373).

The ¹³C NMR together with the DEPT spectrum revealed 30 carbon signals including characteristic signals due to a trisubstituted double bond (δ 127.55 (d) and 140.07 (s)), a carboxylic acid group (δ 180.65), a ketone carbonyl group (δ 216.41), and two alcoholic carbons (δ 69.73 (d) and 72.67 (s)). The ¹H NMR spectrum showed the presence of a hydroxy methine proton (δ 4.80 (1H, dd, J = 12.5, 6.5 Hz), an olefinic proton at δ 5.55 (1H, br s), six singlets at δ 1.01 (3H), 1.09 (3H), 1.15 (3H), 1.22 (3H), 1.42 (3H), 1.64 (3H) for six tertiary methyl group, and a doublet at δ 1.12 (3H, d, J = 6.6 Hz) for a secondary methyl group. The secondary methyl signal on ring E provides a most useful indicator for the presence of an urs-12-ene skeleton. In addition, the signals in its ¹³C NMR at δ 50.10 (C-1) in the HMBC spectrum and a hydroxy methine proton (δ 3.03 (1H, s), H-18) assigned to the urs-12-ene skeleton. In addition, the signals in its ¹³C NMR at δ 127.55 (d) and 140.07 (s) are characteristic for a C-12/C-13 double bond in the ursene-type structure. The presence of the ursene skeleton was further verified using 2D NMR spectroscopy including HMBC measurement. The ¹H–¹³C long-range COSY and ¹H–¹³C long-range COSY spectrum of this compound gave very good information for establishing the assignment of the partial structure of C-1/C-13 carbons. There were long-range correlations between the H-2 proton at δ 4.80 and the methylene carbon at δ 50.10 (C-1) in the HMBC spectrum and a cross-peak between the signals of the H-2 proton and the H-1 proton in the ¹H–¹³C COSY spectrum, which indicated that the ketone carbonyl group must be either at position C-1 or C-3. Furthermore, the observation of the correlation between the ketone carbonyl group and two methyl carbons (C-23 at δ 25.28 and C-24 at δ 21.77) suggested the ketone must be at the C-3 position. The secondary hydroxyl group was assigned to C-2 due to the correlation between the C-1 methylene carbon at δ 50.10 and the C-25 methyl group. Splitting patterns for the hydroxy methine proton (δ 4.80, dd, J = 12.5, 6.5 Hz) showed that the hydroxyl group is equatorial. The stereochemistry of the hydroxyl group was further confirmed by NOE experiments. In particular, on irradiation of the signal of the H-2 proton at δ 4.80, the NOE effect was observed on the H-24 at δ 1.01 and H-25 at δ 1.15 methyl protons. Furthermore, irradiation of the signal of the H-25 methyl protons gave the NOE enhancements for H-26 methyl protons at δ 1.09, H-24 methyl protons, and H-2 proton. These data clearly suggested the equatorial configuration of the C-2 hydroxyl group, as indicated in structure 1. On the other hand, a signal at δ 3.03 (1H, s, H-18) assigned to the bridgehead H-18 suggested the presence of a 19-O-substituted urs-12-ene skeleton. The hydroxyl group at C-19 was assigned to the α position by analogy with the ¹³C NMR chemical shifts of similarly substituted triterpene acids. The carboxylic acid group was assigned to C-17 on the basis of a HMBC correlation to H-18. Furthermore, the structure of compound 1 was supported by analysis of ion peaks in the mass spectrum. The peaks at m/z 246 [264 – H₂O]⁺, 220 [264 – CO₂]⁺, 219 [264 – CO₂H]⁺, and 218 [264 – HCO₂H]⁺ corresponded to the typical retro-Diels–Alder (RDA) cleavage fragments commonly found in the spectra of urs-12-ene derivatives possessing a carboxylic group on C-17 and a hydroxyl substituent on ring D or E. Besides, there was a base peak at m/z 146 apparently due to the secondary retro-Diels–Alder cleavage of the m/z 218 fragment ion. These fragmentation resemble very closely those of tormentic acid. Other important ions were at m/z 438, 366, and 220, which indicated that a secondary hydroxy group and a ketone group were located in the A/B ring portion. The complete assignment of all the carbons was determined (Table 1) with the help of DEPT, ¹H–¹³C COSY, ¹H–¹³C COSY, and NOE and followed by analysis of the HMBC spectral data (Table 2). On the basis of all of the above information, compound 1 was found to be a new triterpene acid, and the structure of the natural product was formulated as 2α,19α-dihydroxy-3-oxo-12-ursen-28-oic acid.

The other known compounds, usric acid (2), epimomal acid (3), maslinic acid (4), 1,11,13 euscaphic acid (5), tormentic acid (6), were identified by comparing their MS and ¹H and ¹³C NMR data with those reported in the literature. They were further confirmed by 2D NMR spectra. Among these known triterpene acids, maslinic acid and tormentic acid have previously been reported to be present in G. japonicum.

All the compounds isolated from the EtOAc-soluble fraction were tested for their anti-HIV-1 protease activity by HPLC assay. 2α,19α-Dihydroxy-3-oxo-12-ursen-
28-oic acid (1) (72%), ursolic acid (2) (85%), and maslinic acid (4) (100%) exhibited stronger activity than the others [epipomolic acid (3) (42%) and tormentic acid (6) (49%), respectively] at the concentration of 17.9 μg/mL. Maslinic acid (4) was the most active, while eusapnic acid (5) (0%) did not show any activity at the tested concentration. This is the first report of anti-HIV-1 activity of this plant. Further studies of structure--activity relationships are currently in progress in our laboratory, but the results of the experiments obtained in the present study indicate that compounds 1, 2, and 4 may contribute toward the anti-HIV-1 protease activity of the EtOAc-soluble fraction of G. japonicum.

Experimental Section

General Experimental Procedures. Optical rotations were obtained on a Perkin automatic polarimeter. IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR spectrometer. EIMS were recorded on a MICRO-MASS 7035E mass spectrometer at 70 eV. All NMR spectra (1H, 13C, COSY, NOE, HMGC and HMQC) were recorded on a Bruker AMX 500 spectrometer (500 MHz for 1H and 125 MHz for 13C) in pyridine-d$_5$. Optical rotations were recorded on a Perkin-Elmer 1600 Series IR spectrometer. EIMS were recorded on a MICRO-MASS 7035E mass spectrometer at 70 eV. All NMR spectra (1H, 13C, COSY, NOE, HMGC and HMQC) were recorded on a Bruker AMX 500 spectrometer (500 MHz for 1H and 125 MHz for 13C) in pyridine-d$_5$. The chemical shifts are reported in ppm with TMS as an internal standard, and coupling constants (J) are given in Hz. TLC was done on 0.25 mm silica gel (60 F254, Merck) plates.

Plant Material. The whole plant of G. japonicum used in this experiment was collected from China in Aug 1994. The material was identified as G. japonicum Thunb. by Dr. Dao-Feng Chen, Department of Pharmacology, Shanghai Medical University, China. A voucher specimen is deposited in Department of Pharmacology, Shanghai University of Traditional Chinese Medicine, China.

Extraction and Isolation. Dried whole plants (2.6 kg) were chopped into small pieces and extracted three times with MeOH (20 L) at room temperature for 7 days, and the extract was evaporated in vacuo to yield MeOH extract (350 g). The MeOH extract was suspended in distilled water (1 L) and successively extracted with hexane (2 L × 5) and EtOAc (2 L × 5). The EtOAc-soluble fraction was filtered, and the filtrate was evaporated under reduced pressure to give a brown oil (120 g).

The EtOAc extract (100 g) was subjected to silica gel column chromatography using a linear gradient CHCl$_3$–MeOH system. The fractions with CHCl$_3$–MeOH 9:1–7:3 were further purified by repeated silica gel column chromatography and/or preparative TLC to give 2a,19α-dihydroxy-3-oxo-12-ursen-28-oic acid (1) (44 mg), 3β-hydroxy-12-ursen-28-oic acid (2) (70 mg), 3α,19α-dihydroxy-12-ursen-28-oic acid (3) (23 mg), 2α,3β,20α-trihydroxy-12-ursen-28-oic acid (4) (30 mg), 3α,20α-trihydroxy-12-ursen-28-oic acid (5) (50 mg), and 2α,3β,19α-trihydroxy-12-ursen-28-oic acid (6) (20 mg).

2α,19α-Dihydroxy-3-oxo-12-ursen-28-oic acid (1): colorless amorphous powder, $[α]_D +1.7^°$ (c 1.0, EtOH); mp 215–217 °C; HREIMS 486.3373 (calcld for C$_{30}$H$_{46}$O$_5$, 486.3345); EIMS m/z 486 (M$^+$, 0.8), 438 (5.8), 366 (34.6), 246 (10.7), 219 (9.9), 185 (32.5), 146 (100); IR KBr (cm$^{-1}$) 3600–3200, 2928, 2869, 1684, 1650, 1384, 1105; 1H NMR (pyridine-d$_5$, 500 MHz) $\delta$ 1.01 (3H, s, 24-H$_3$), 1.09 (3H, s, 26-H$_3$), 1.12 (3H, d, $J = 6.6$ Hz, 30-H$_3$), 1.15 (3H, s, 25-H$_3$), 1.22 (3H, s, 23-H$_3$), 1.25 (1H, m, 5-H), 1.37 (1H, m, 1-H$_3$), 1.42 (3H, s, 29-H$_3$), 1.50 (1H, m, 20-H), 1.64 (3H, s, 27-H$_3$), 1.87 (1H, m, 7 = 8.9 Hz, 9-H), 2.08 (1H, m, 21-H$_3$), 2.11 (1H, m, 22-H$_3$), 2.13 (1H, m, 21-H$_3$), 2.15 (1H, m, 22-H$_3$), 2.48 (1H, dd, $J = 12.5$, 6.5 Hz, 1-H$_3$), 3.03 (1H, s, 18-H), 4.80 (1H, dd, $J = 12.5$, 6.5 Hz, 2-H), 5.55 (1H, br s, 12-H): 13C NMR (pyridine-d$_5$) see Table 1.

Biological Assay. Recombinant HIV-1 protease was obtained from the expression vector, PEGX-PR 107, in Escherichia coli DH5α and was purified according to the method established in our laboratory.15

The proteolytic activity of HIV-1 PR was measured using the synthetic heptapeptide Ser-Gln-Asn-Tyr-Pro-Arg-Lys (SONYPYIV), corresponding to the p24–p17 cleavage site in the natural gag precursor, as substrate. The products of cleavage were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC, Ultrasphere ODS, 5 μm, 4.6 mm × 15 cm, Beckman, CA) with a 7–33% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA). Product peak areas were integrated by a data system (Chromatopac C-R3A, Shimadzu, Japan) and compared with the standard peptide SQNY, which is expected to be generated from heptapeptide SQNYPIV after HIV-1 protease digestion. The extinction coefficient of the peptide SQNY was used to estimate the rate of proteolysis of the heptapeptide substrate. The assay was performed in a volume of 140 μL containing 90 pmol of substrate, 2 U of HIV-1 protease, and compound solution in 0.1 M NaOAc buffer, pH 5.5, with 1 M (NH$_4$)$_2$SO$_4$ at 37 °C. The reaction, which proceeded for 2 h, was terminated by the addition of 20 μL of aqueous 10% trifluoroacetic acid. The supernatant obtained by centrifugation at 12 000 rpm for 3 min was then analyzed by the HPLC method. The unit of enzyme activity was defined as the amount of enzyme which yields 1 nmol of tetrapeptide SQNY under the above conditions in 1 min.

Proteolytic activity of HIV-1 protease inhibited by the compound was calculated as the activity of the control subtracting that of the sample and then divided by itself.

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References and Notes