A New Anti-HIV Triterpene from *Geum japonicum*

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Geumonoid (1), a new triterpene, was isolated from *Geum japonicum*. Its structure was elucidated on the basis of 1D, 2D NMR and MS spectroscopic analysis. Compound 1 showed inhibitory activity against HIV-1 protease.

Key words *Geum japonicum*; triterpene; geumonoid; anti-HIV protease; Rosaceae

Previous phytochemical studies on the constituents of *Geum japonicum* Thunb. (Rosaceae) led to the discovery of many compounds, including triterpenoids and tannins.[1—6] As part of our continuous searching for novel bioactive agents from medicinal plants, a methanol extract from the whole plant of *G. japonicum* was found to show significant inhibitory activity against HIV-1 protease. Bioactivity-guided chromatographic fractionation of the active extract led to the isolation and characterization of a new compound, named geumonoid (**1**). Herein, we report the isolation and structural elucidation of the new compound and its inhibitory activity against HIV-1 protease.

Geumonoid (**1**), obtained as an amorphous powder, showed a molecular peak at *m/z* 486 $[M]^+$ in its EIMS. A molecular formula $C_{30}H_{46}O_5$ was determined from comprehensive examination of the spectral data (EI-MS and NMR) and elemental analysis. It gave a positive response to the Liebermann–Burchard reagent. Its IR spectrum showed the presence of a carbonyl (1714 cm^{-1}), a carboxyl (1700 cm^{-1}), and hydroxyl (3424 cm^{-1}) groups. The ^{1}H-NMR spectrum of **1** showed signals for seven methyl groups at δ 0.89, 0.99, 1.05, 1.06, 1.07, 2.11 (each 3H, s), and 1.26 (d, J=6.8 Hz); two oxymethine groups at δ 4.15 (ddd, J=11.2, 9.3, 4.4 Hz) and 3.40 (d, J=9.3 Hz); three olefinic protons at δ 5.77 (1H, d, J=10.1 Hz), 6.16 (1H, dd, J=10.1, 2.8 Hz), and 5.85 (1H, s). The coupling constant of proton signals at δ 5.77 and 6.16 is 10.1, suggesting the presence of two *cis*-olefinic protons. The olefinic proton at δ 5.85 occurred as a singlet, indicating that no proton was affixed to the adjacent carbon.

The ^{13}C-NMR spectrum and DEPT experiments showed 30 carbon signals: seven methyl, seven methylene, eight methine (including three olefinic methine and two oxymethine), and eight quaternary (including an olefinic, a carboxylic, and a carbonyl) carbons. The carbonyl, carboxyl and olefinic groups account for four of the eight units of unsaturation required by the molecular formula, and thus, compound **1** was determined to be tetracyclic, which suggested that this compound might be a triterpene.

The HMQC spectrum established the one bond correlation between carbons and hydrogens in compound **1**. The connections of the groups were derived from ^{1}H–^{1}H COSY NMR and HMBC spectra. In the ^{1}H–^{1}H COSY NMR spectrum, the doublet at δ 3.40 (H-3) showed a cross peak with δ 4.15 (ddd) attributed to the H-2 proton. The latter was coupled with two multiplets at δ 2.50 (1H) and 1.36 (1H), assigned as the two protons (H-1) of a methylene. The cross peaks between δ 1.10 (H-5) and 1.44 (H-6a), 1.44 (H-6a) and 1.63 (H-6b), 1.63 (H-6b) and 2.18 (H-7a), 2.18 (H-7a) and 1.25 (H-7b) were also observed in the ^{1}H–^{1}H COSY NMR spectrum. The above correlations revealed the connectivities of CH (3)–CH (2)–CH_2 (1) and CH (5)–CH_2 (6)–CH_2 (7). The HMBC spectrum of **1** (see Fig. 1) revealed the long-range correlations from δ 55.43 (C-5) to δ 1.07 (H-24), and 0.99 (H-25); from δ 83.77 (C-3) to δ 2.50, 1.36 (H-1); from δ 47.42 (C-9) to δ 2.18, 1.25 (H-7), and 1.10 (H-5); and from δ 211.40 (C-11) to δ 2.11 (H-12), and δ 2.52 (H-9). This information, together with the EI-MS fragments (see Fig. 2) at *m/z* 281, 279, 263, 261, 169, 167, 149, and 43, suggest the presence of partial structure A in compound **1**.

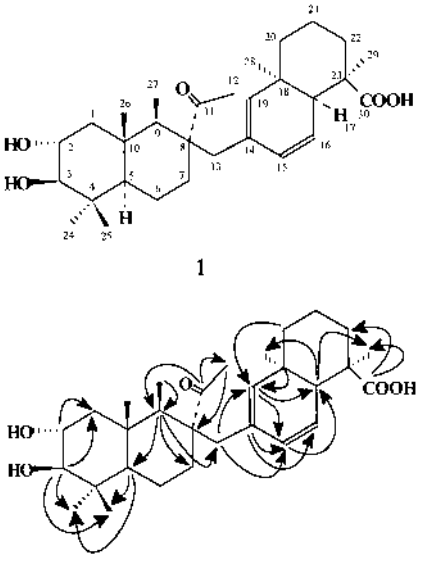

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Fig. 1. Selective HMBC Observations for Geumonoid
Arrows denote HMBC correlation from C to H.

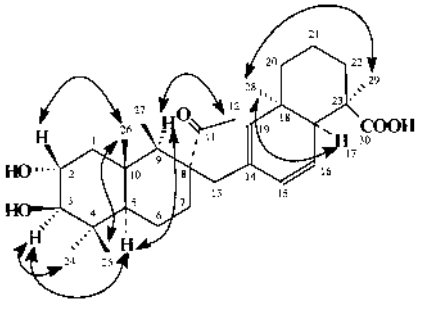

Fig. 2. NOE Interactions Obtained in the NOESY Spectrum of **1**

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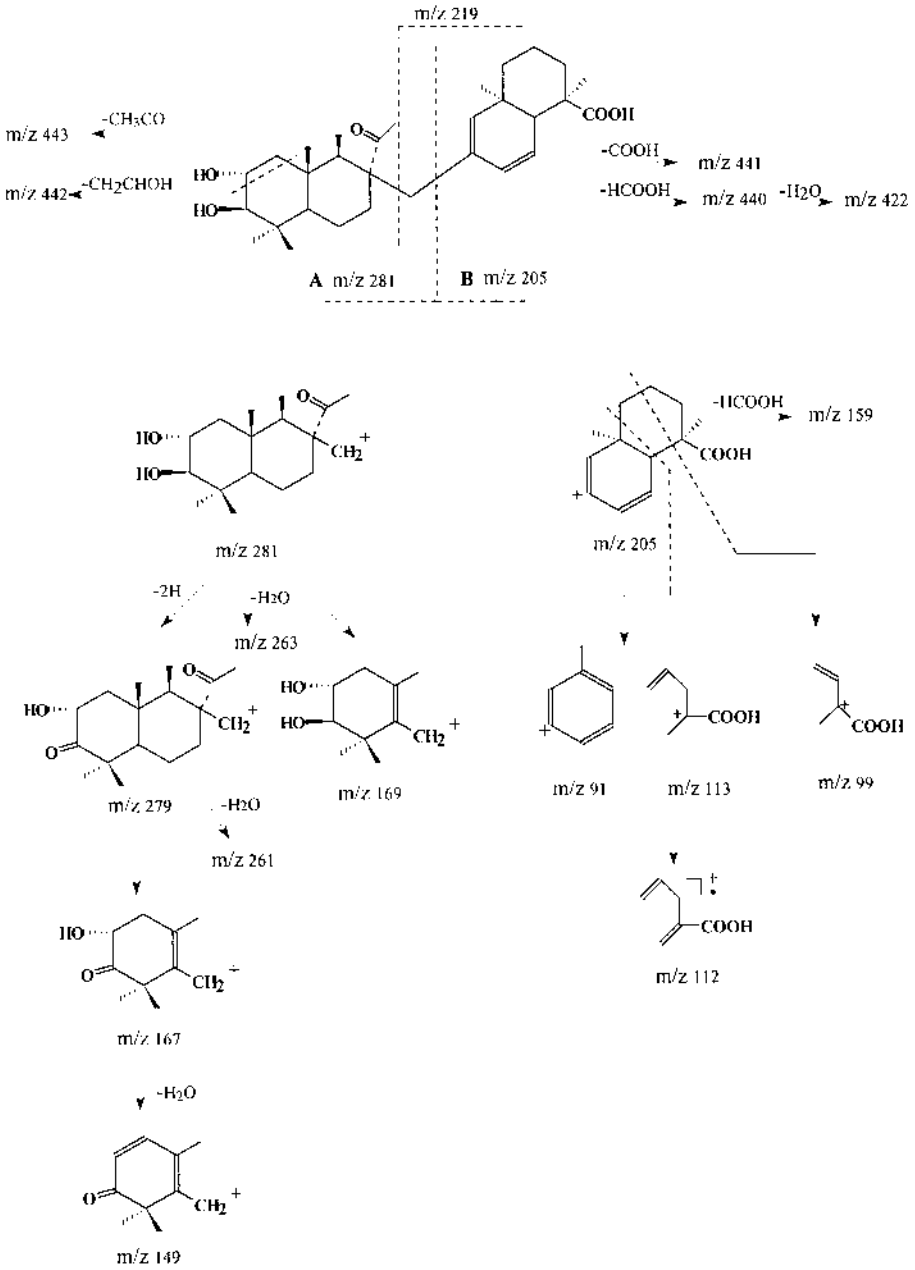

Fig. 3. The EIMS Fragments of **1**

In addition to the above data, the correlations between δ 5.77 (d, J=10.1 Hz, H-15) and 6.16 (dd, J=10.1, 2.8 Hz, H-16) and 2.17 (d, J=2.8 Hz, H-17), δ 2.57 (m, H-20a) and 1.64 (m, H-20b), δ 1.64 (m, H-20b) and 2.01 (m, H-21a), δ 2.01 (m, H-21a) and 1.65 (m, H-21b), 1.65 (m, H-21b) and 1.38 (m, H-22a) and 1.34 (m, H-22b) were also observed in the ^{1}H–^{1}H COSY NMR spectrum, and the long-range correlations from H-16 to C-14, C-17 and C-23; from H-15 to C-13, C-17, and C-19; from H-19 to C-13, C-18, and C-20; from C-17 to H-28, H-29; from C-30 to H-22, H-29; and from C-13 to H-15 were observed in the HMBC spectrum of **1** (see Fig. 1). This information, together with the EI-MS fragments (see Fig. 3) at *m/z* 219, 205, 113, 112, 99, implied partial structure B existing in the molecule of compound **1** and determined the linkage position of the partial structures of A and B.

The C-3 proton appeared to be a doublet with a coupling constant of 9.3 Hz, which suggested axial–axial couplings between C-2H and C-3H. Comparing the chemical shift values of carbon signals at C-2 and C-3 (δ 68.56, 83.77) with those of the known compound tormentic acid[7] suggested that they have the same configurations at C-2 and C-3. Thus, the configurations of protons at C-2 and C-3 were deduced to be β and α, respectively, which were further confirmed by the NOE interactions in the NOESY spectrum of **1** (see Fig. 2). The cross peaks observed in the NOESY spectrum between H-3 and H-5, H-3 and H-24, H-5 and H-9, H-2 and H-26, H-26 and H-27 implied that the relative configurations of H-2, H-5, H-9, 9-methyl and 10-methyl were α, α, α, β, and β, respectively. The NOESY spectrum also showed cross peaks between H-17 and H-28, and between H-28 and H-29. Thus, the orientation between H-17, 18-methyl, and 23-methyl are all *cis* in the opposite direction to the carboxylic group. The above information enabled us to determine the structure and

Table 1. NMR Data for Geumonoid (in Pyridine-d_5)

Position	δ_H	δ_C
1	2.50 m, 1.36 m	47.34 (t)
2	4.15 ddd (11.2, 9.3, 4.4)	68.56 (d)
3	3.40 d (9.3)	83.77 (d)
4		39.85 (s)
5	1.10 m	55.43 (d)
6	1.44 m, 1.63 m	18.65 (t)
7	2.18 m, 1.25 m	26.60 (t)
8		40.88 (s)
9	2.52 s	47.42 (d)
10		38.19 (s)
11		211.40 (s)
12	2.11 s	28.04 (q)
13	2.01 m, 1.70 m	38.97 (t)
14		142.45 (s)
15	5.77 d (10.1)	127.41 (d)
16	6.16 dd (10.1, 2.8)	130.55 (d)
17	2.17 d (2.8)	54.65 (d)
18		41.53 (s)
19	5.85 s	128.98 (d)
20	2.57 m, 1.64 m	27.42 (t)
21	1.38 m, 1.34 m	32.50 (t)
22	2.01 m, 1.65 m	28.16 (t)
23		47.53 (s)
24	1.07 s	16.33 (q)
25	0.99 s	19.39 (q)
26	1.05 s	17.08 (q)
27	1.26 d (6.8)	28.99 (q)
28	1.06 s	20.16 (q)
29	0.89 s	16.82 (q)
30		177.87 (s)

relative stereochemistry of this molecule, as shown in **1**.

Compound **1** was tested for HIV-1 protease inhibitory effects with a recombinant enzyme, which exhibited strong activity with 89% inhibition at the concentration of 17.9 μg/ml. The studies of the structure–activity relationships and action model of the compound are currently in progress in our laboratory.

Experimental

General Melting points were measured with an XT4-100x micro-melting point apparatus and are uncorrected. An optical rotation was obtained on a Perkin-Elmer 241 polarimeter. The IR spectrum was recorded on a Perkin-Elmer 1600 series FT-IR spectrophotometer. NMR spectra were run on a Bruker AM-500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). EIMS was recorded on a Micro-Mass 7035E Mass spectrometer at 70 eV. Elemental analysis was performed on a MoD1106 elemental analyzer. Silica gel 60 (Merck 100—200 mesh) was used for column chromatography. Precoated silica gel Kieselgel 60 F_{254} plates (0.25 mm thickness) were used for TLC, and the spots were detected by spraying with 25% phosphomolybdic acid, followed by heating. Merck silica gel 60 F_{254} was used for preparative thin layer chromatography (PTLC, 0.5 mm thickness).

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 Pharmacognosy, Shanghai Medical University, China. A voucher specimen was deposited in the Department of Pharmacognosy, 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 d; then 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. The EtOAc extract (100 g) was subjected to silica gel column chromatography using a linear gradient with a $CHCl_3$–MeOH system. The fractions with $CHCl_3$: MeOH (8 : 2) were further purified by repeated silica gel column chromatography and preparative TLC to give geumonoid (**1**, 10 mg).

Geumonoid (**1**): An amorphous powder, $[\alpha]_D^{25}$ +10.1° (c=1.0, EtOH); IR (KBr) ν_{max} cm^{-1}: 3424, 2934, 1714, 1700, 1654, 1636, 1560, 1458, 1384, 1048; ^{13}C- and ^{1}H-NMR data, see Table 1; EI-MS *m/z*: 486 (20), 468 (60), 443 (25), 442 (70), 441 (15), 440 (30), 422 (20), 281 (5), 279 (20), 263 (15), 261 (20), 219 (10), 205 (40), 169 (20), 167 (65), 159 (28), 149 (90), 113 (25), 112 (20), 99 (55), 91 (40), 43 (100). *Anal.* C, 74.12; H, 9.45, Calcd for $C_{30}H_{46}O_5$: C, 74.07; H, 9.47.

Biological Assay Recombinant HIV-1 protease was obtained from the expression vector, PGEX-PR 107, in *Escherichia coli* DH5a and was purified according to the method in literature.[8] The proteolytic activity of HIV-1 PR was measured using the synthetic heptapeptide Ser–Gln–Asn–Tyr–Pro–Ile–Val (SANYPIV), corresponding to the p24–p17 cleavage site in the natural gag precursor, as a substrate. The products of cleavage were analyzed by HPLC (Ultrasphere ODS, 5 μm, 4.6 mm×15 cm, Beckman, CA, U.S.A.) 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 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 $(NH_4)_2SO_4$, at 37 °C. The reaction, processed for 2 h, was terminated by the addition of 20 μl of aqueous 10% trifluoroacetic acid. The supernatant obtained by centrifugation at 12000 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.

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

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