New Glabretal Triterpenes from the Immature Fruits of Poncirus trifoliata and Their Selective Cytotoxicity

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Note

Two new glabretal triterpenes, pancastatins A (1) and B (2), were isolated from the immature fruits of Poncirus trifoliata. Their chemical structures were elucidated by spectroscopic analyses including one- and two-dimensional NMR and high-resolution electrospray ionization mass spectrometry. Compounds 1 and 2 exhibited selective cytotoxicity against PANC-1 pancreatic cancer cells under low-glucose stress conditions.

Key words Poncirus trifoliata; pancastatin; glabretal triterpene; selective cytotoxicity

Treatment for pancreatic cancer includes anti-cancer chemotherapy and radiotherapy. The anti-cancer drug gemcitabine is showing greater effect than others, but the reaction rate and survival rate are still low. These anti-cancer drugs are not selective to cancer and lead to side effects, such as vomiting, mucosal ulcers, and alopecia.1,2 Therefore, there is a need to develop an anti-cancer drug that targets cancer cells and reduces side effects by only affecting pancreatic cancer cells without damaging normal cells.3,4 The typical pancreatic cancer solid tumor is often exposed to stress conditions, such as glucose deprivation.5,6 When stress-related molecular chaperone glucose-regulated protein (GRP)78 is over expressed causing pancreatic cancer cells to acquire strong resistance to drugs and consequently continue to survival.7,8

In order to prevent the side effects of anti-cancer drugs and develop an effective pancreatic cancer drug, this study explores a substance with more selective anti-cancer activity in the glucose deprivation state caused by 2-deoxyglucose (2-DG) than in the normal state. We isolated pancastatins A (1) and B (2) from the immature fruits of Poncirus trifoliata.

The dried immature fruits of Poncirus trifoliata (Rutaceae) are widely used as a traditional herbal medicine in Eastern Asia to ameliorate gastritis, ulcers, and other inflammation-related diseases.9,10 This plant is known to contain a variety of constituents, such as coumarins, flavonoids, terpenoids, and essential oils.11 Also, the extracts and constituents of Poncirus trifoliata exhibit diverse biological properties, including anti-oxidant, anti-platelet, anti-bacterial, and anti-allergic activities.12,13 Here, we report the isolation and structure determination of two new glabretal triterpenes, pancastatins A (1) and B (2) (Chart 1), and describe their biological properties. Glabretal triterpenes usually occur as epimeric mixtures with different ratios because of the presence of a hemiacetal moiety in their structures.14–16 From 1H-NMR spectral data, pancastatin A is a mixture of epimers with a ratio of 1 : 0.7 and pancastatin B is a mixture of epimers with a ratio of 1 : 0.9. Therefore the structural elucidation was carried out with the major component.

The methanolic extract of the immature fruits of P. trifoliata was suspended in H₂O, and partitioned with hexane and ethyl acetate. The ethyl acetate-soluble portion was fractionated via open-column chromatography on silica gel, Sephadex LH-20, and a RP-sepak cartridge, and was subjected to semi-preparative HPLC to yield compounds 1 and 2.

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Compound 1 was isolated as a white powder and exhibited a UV spectrum with $\lambda_{\text{max}}$ at 267 nm. It exhibited a molecular ion peak at $m/z$ 677.3 [M+Na]$^+$ in the electrospray ionization (ESI)-MS, suggesting a molecular weight of 654; the molecular formula was determined as C$_{40}$H$_{62}$O$_7$ by high-resolution (HR)-ESI-MS ($m/z$ 677.4385 [M+Na]$^+$). The 1H-NMR spectrum of 1 (Table 1) showed signals due to six tertiary methyls at $\delta$ 0.84, 0.90, 0.92, 0.96, 1.07, and 1.27, four olefinic methines at $\delta$ 5.90, 5.95, 6.20, and 7.67, and four oxygenated methines at $\delta$ 3.02, 3.74, 3.81, and 4.69. Further, the signals of one cyclopropyl methylene at $\delta$ 0.52 and 0.70, one hemiacetal methine at $\delta$ 5.34, and one oxygenated methylene at $\delta$ 3.50/3.45 were evident. The remaining signals indicated twelve methylenes and four methines in compound 1. In the 13C-NMR spectrum, one carbonyl carbon at $\delta$ 167.2, six methyl carbons at $\delta$ 13.1, 13.4, 15.0, 19.1, 20.9, and 26.9, four olefinic methine carbons at $\delta$ 121.2, 126.3, 139.5, and 141.4, four oxygenated methine carbons at $\delta$ 64.4, 74.1, 78.3, and 78.3, one hemiacetal carbon at $\delta$ 97.9, one oxygenated methylene carbon at $\delta$ 65.8, one oxygenated quaternary carbon at $\delta$ 60.1, and several carbon signals for methine and methylene between $\delta$ 13.1 and 50.4 were present in compound 1. The distortionless enhancement by polarization transfer (DEPT) spectrum indicated that 40 carbons comprised six methyl, 14 methylene, 13 methine, and seven quaternary carbons. As above-described evidence, 1H- and 13C-NMR observation were very similar to previously published data for glabretal-type triterpene, suggesting that compound 1 was a glabretal-type triterpene. The 1H–1H correlation spectroscopy (1H–1H COSY) spectrum of 1 revealed five partial structures including a 2,4-decadienoyl moiety, as shown in Fig. 1, and all proton-bearing carbons were verified by heteronuclear multiple quantum coherence (HMOC).

### Table 1. 1H- and 13C-NMR Spectral Data of 1

| No. | Major epimer | Minor epimer |
|-----|--------------|--------------|
| 1   | 33.9         | 33.8         |
| 2   | 22.6         | 22.6         |
| 3   | 78.3         | 78.3         |
| 4   | 36.3         | 36.3         |
| 5   | 41.1         | 41.2         |
| 6   | 37.2         | 37.0         |
| 7   | 24.5         | 24.5         |
| 8   | 74.1         | 74.0         |
| 9   | 39.0         | 39.1         |
| 10  | 44.4         | 44.2         |
| 11  | 16.3         | 16.2         |
| 12  | 25.8         | 25.7         |
| 13  | 28.8         | 28.2         |
| 14  | 37.2         | 36.1         |
| 15  | 25.8         | 25.4         |
| 16  | 27.2         | 27.7         |
| 17  | 44.8         | 48.6         |
| 18  | 13.8         | 13.6         |
| 19  | 15.0         | 14.9         |
| 20  | 49.4         | 50.4         |
| 21  | 97.9         | 102.0        |
| 22  | 30.3         | 32.3         |
| 23  | 78.3         | 76.9         |
| 24  | 64.4         | 62.2         |
| 25  | 60.1         | 59.9         |
| 26  | 13.4         | 13.6         |
| 27  | 65.8         | 65.6         |
| 28  | 26.9         | 26.9         |
| 29  | 20.9         | 20.9         |
| 30  | 19.1         | 19.0         |

a) 1H- and 13C-NMR spectra were recorded at 600 and 150MHz in CD$_3$OD, respectively. b) Proton resonance multiplicity and coupling constants in parentheses.
spectrum. The structure was elucidated by heteronuclear multiple-bond coherence (HMBC) spectrum. In the HMBC spectrum, the methine protons at $\delta 4.69, 5.95$, and $7.67$ showed long-range correlations to a carbonyl carbon at $\delta 167.2$, revealing that the 2,4-decadienoic ester was connected to C-3. The long-range correlations from the methyl proton at $\delta 0.84$ and $0.92$ to carbons at $\delta 36.3, 41.1$, and $78.3$, from the methyl protons at $\delta 0.96$ to carbons at $\delta 37.2, 33.9, 41.1$, and $44.4$, from the methyl protons at $\delta 1.07$ to carbons at $\delta 37.2, 39.0, 44.4$, and $74.1$ established the structure of A and B rings of triterpene skeleton. The correlations from methylene protons at $\delta 0.70/0.52$ to carbons at $\delta 25.8, 28.8, 37.2, 39.0$, and $44.8$ revealed the presence of cyclopropyl moiety and established the structure of the C and D rings of triterpene skeleton. Also, the long-range correlations from the methyl protons at $\delta 1.27$ to oxygenated carbons at $\delta 60.1, 64.4$, and $65.8$, and from oxy-

![Fig. 1. Structures of Pancastatins A (1) Elucidated by Two-Dimensional NMR Experiments](image)

Table 2. $^1$H- and $^{13}$C-NMR Spectral Data of 24

| No. | $\delta$C | $\delta$H | $\delta$C | $\delta$H |
|-----|-----------|-----------|-----------|-----------|
| 1   | 33.9      | 1.42, 1.24| 33.8      | 1.42, 1.24|
| 2   | 22.6      | 1.58, 1.99| 22.6      | 1.58, 1.99|
| 3   | 78.4      | 4.70      | 78.4      | 4.70      |
| 4   | 36.3      |           |           |           |
| 5   | 41.1      | 2.08      | 41.2      | 2.08      |
| 6   | 37.2      |           | 37.0      |           |
| 7   | 24.5      | 1.61, 1.68| 24.5      | 1.61, 1.68|
| 8   | 74.1      | 3.74      | 74.0      | 3.74      |
| 9   | 39.0      |           | 39.1      |           |
| 10  | 44.4      | 1.41      | 44.2      | 1.41      |
| 11  | 16.3      | 1.41, 1.33| 16.3      | 1.41, 1.33|
| 12  | 25.8      | 1.76, 2.12| 25.7      | 1.91, 1.99|
| 13  | 28.8      |           | 28.2      |           |
| 14  | 37.2      |           | 36.1      |           |
| 15  | 25.8      | 1.61, 1.92| 25.4      | 1.61, 1.92|
| 16  | 26.9      | 0.98, 1.63| 27.3      | 0.98, 1.63|
| 17  | 44.8      | 2.14      | 48.5      | 2.00      |
| 18  | 13.6      | 0.51, 0.70| 13.5      | 0.50, 0.74|
| 19  | 15.0      | 0.96      | 14.9      | 0.96      |
| 20  | 49.4      | 1.84      | 50.4      | 2.06      |
| 21  | 97.9      | 5.34 (d, $J=4.1$ Hz) | 102.0 | 5.29 (d, $J=4.1$ Hz) |
| 22  | 30.3      | 1.65, 2.00| 32.3      | 1.46, 2.07|
| 23  | 78.3      | 3.81      | 76.9      | 3.93      |
| 24  | 64.4      | 3.02 (d, $J=8.3$ Hz) | 62.2 | 2.89 (d, $J=7.6$ Hz) |
| 25  | 60.1      |           | 59.9      |           |
| 26  | 13.4      | 1.27      | 13.6      | 1.28      |
| 27  | 65.8      | 3.50, 3.45| 65.6      | 3.50, 3.48|
| 28  | 26.9      | 0.85      | 26.9      | 0.85      |
| 29  | 20.9      | 0.93      | 20.9      | 0.93      |
| 30  | 19.0      | 1.07      | 18.9      | 1.06      |
| 1'  | 167.1     |           | 167.2     |           |
| 2'  | 121.6     | 5.98 (d, $J=15.1$ Hz) | 121.6 | 5.98 (d, $J=15.1$ Hz) |
| 3'  | 139.0     | 7.71 (dd, $J=15.1, 11.7$ Hz) | 139.0 | 7.72 (dd, $J=15.1, 11.7$ Hz) |
| 4'  | 126.0     | 6.19 (dd, $J=11.7, 10.3$ Hz) | 126.0 | 6.19 (dd, $J=11.7, 10.3$ Hz) |
| 5'  | 139.2     | 5.84 (dt, $J=10.3, 7.6$ Hz) | 139.2 | 5.84 (dt, $J=10.3, 7.6$ Hz) |
| 6'  | 26.0      | 3.08      | 26.0      | 3.08      |
| 7'  | 125.2     | 5.34 (dt, $J=10.3, 7.6$ Hz) | 125.2 | 5.34 (dt, $J=10.3, 7.6$ Hz) |
| 8'  | 132.8     | 5.46 (dt, $J=10.3, 7.6$ Hz) | 132.8 | 5.46 (dt, $J=10.3, 7.6$ Hz) |
| 9'  | 20.2      | 2.12      | 20.2      | 2.12      |
| 10' | 13.3      | 0.98      | 13.3      | 0.98      |

$^a$) $^1$H- and $^{13}$C-NMR spectra were recorded at 600 and 150MHz in CD$_3$OD, respectively. $^b$) Proton resonance multiplicity and coupling constants in parentheses.
genated methylene protons at $\delta$ 3.50/3.45 to carbons at $\delta$ 13.4, 60.1, and 64.4 estimated the existence of the oxiran moiety in the structure of I. Finally the tetrahydrofuran moiety was established on the basis of the correlation a methine proton at $\delta$ 3.81 and a hemiacetal carbon at $\delta$ 97.9. The stereochemistry of I was established by the nuclear Overhauser effect spectroscopy (NOESY) spectrum, which showed the NOEs between $\delta$ 4.69 and $\delta$ 1.99/1.58/0.92, between $\delta$ 1.99 and $\delta$ 0.92, between $\delta$ 2.08 and $\delta$ 1.61/1.23/0.84, between $\delta$ 1.68 and $\delta$ 3.74/1.07, between $\delta$ 1.07 and $\delta$ 1.92, between $\delta$ 5.34 and $\delta$ 2.15/1.84, and between $\delta$ 3.02 and $\delta$ 3.50/3.45/1.65. The stereochemistry of I resembled those of glabratel-type triterpenoids previously reported.\textsuperscript{14–16} The geometry of two double bonds of 2,4-decadienoic ester group were trans, and named as pancastatin A. A minor component was an epimer with different stereochemistry at the C-21 position.

Compound 2 was isolated as a white powder and exhibited a UV spectrum with $\lambda_{max}$ at 267 nm. It exhibited a molecular ion peak at $m/z$ 675.3 [M+Na]\textsuperscript{+} in the ESI-MS, suggesting a molecular weight of 652. The molecular formula was determined as $C_{40}H_{60}O_{7}$ by high resolution ESI-MS ($m/z$ 675.4243 [M+Na]\textsuperscript{+}). Both the $^1$H- and $^{13}$C-NMR spectra of 2 (Table 2) were very similar to I, suggesting that compound 2 was also a glabratel-type triterpene. A comparison of the $^1$H- and $^{13}$C-NMR spectra of 1 and 2 showed that the triterpene skeleton of 2 was in good agreement with that of 1. However, the acyl moiety attached to C-3 was different from compound 1. The structure of the acyl side chain was assigned as a 2,4,7-decatrienoyl group by two-dimensional NMR study including mainly $^1$H–$^1$H COSY, which showed the correlations from H-2’ up to H-10’. The 2,4,7-decatrienoyl group was attached to C-3 on the basis of the long-range correlation from the methine proton $\delta$ 4.70 to the carbonyl carbon at $\delta$ 167.1. The configurations of three double bonds were established as trans, cis, and cis from the coupling constants of 15.1, 10.3, and 10.3Hz, respectively. In conclusion, the structure of 2 was determined as a new glabratel-type triterpene containing a 2,4,7-decatrienoyl group in the C-3 position, and named as pancastatin B. A minor component was an epimer with different sterechemistry at C-21 position.

To investigate the cytotoxicity of pancastatins A (1) and B (2), we examined cell viability of PANC-1 cells using the metabolized from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. PANC-1 cells have a typical solid cancerous form and can be found in vivo in low-glucose, high-stress microenvironments. The cells were exposed to the indicated various concentrations (1, 5, 10 $\mu$M) of 1 and 2 under both normal and glucose deprivation conditions. Under normal growth condition, pancastatins A (1) and B (2) treatment of PANC-1 cells had only a weak effect on cell viability. But the 1 and 2 treatment under low-glucose stress condition exhibited selective highly cytotoxicity against PANC-1 cells with IC_{50} values of 3.17 and 2.97 $\mu$M, respectively (Fig. 2).

**Experimental**

**General Experimental Procedures** $^1$H-NMR (600 MHz), $^{13}$C-NMR (150 MHz), and two-dimensional (2D) NMR spectra were obtained on a JEOL JNM-ECA600 spectrometer, with methanol-d$_4$ (CD$_3$OD) as a solvent. HR-ESI-MS was conducted using a Shimadzu LCMS-IT-TOF mass spectrometer. The HPLC system consisted of a HITACHI L-2130 pump, a HITACHI UV detector L-2400, and a reversed-phase column (C18, 5 $\mu$m, $\phi$10×150 mm, Cosmosil). Reversed-phase column chromatography was conducted using RP-C$_{18}$ silica gel (YMC*GEL ODS-A, 12 nm S-150 $\mu$m, YMC Co., Ltd.), and silica gel column chromatography was conducted using Kieselgel 60 (70–230 and 200–400 mesh, Merck). TLC was conducted using Kieselgel 60F$_{254}$ plates (Merck).

**Plant Material** The dried immature fruits of *Poncirus trifoliata* were purchased at an herbal drug store at Keumsan, Korea. A voucher specimen was deposited in the herbarium of the Laboratory of Natural Products Chemistry, Chonbuk National University.

**Extraction and Isolation** The immature fruits of *P. trifoliata* (1 kg) were extracted with methanol at room temperature for 1d. The methanolic extract was concentrated under reduced pressure to give a residue. The residue was suspended in distilled H$_2$O (2 L) and extracted with hexane and ethyl acetate. The ethyl acetate-soluble portion was subjected to silica gel (230–400 mesh, Merck, $\phi$10×17 cm) column chromatography using a stepwise solvent system of chloroform–methanol (100:1–20:1, v/v). An active fraction was re-chromatographed on a silica gel column ($\phi$5×11 cm) eluted with chloroform–methanol (50:1, v/v). Active fractions were combined, concentrated, and chromatographed on a Sepha-
Pancastatin A (1): White powder; UV $\lambda_{max}$ (MeOH) 267 nm; IR $\nu_{max}$ 3440, 2920, 1630, 1380, 1020 cm$^{-1}$; For $^1$H-NMR and $^{13}$C-NMR, see Table 1; ESI-MS (positive mode) $m/z$ 677.3 [M+Na]$^+$; HR-ESI-MS $m/z$ 677.4385 [M+Na]$^+$ (Calcd for C$_{40}$H$_{62}$O$_7$Na, 677.4393).

Pancastatin B (2): White powder; UV $\lambda_{max}$ (MeOH) 267 nm; IR $\nu_{max}$ 3420, 2920, 1640, 1440, 1380, 1020 cm$^{-1}$; For $^1$H-NMR and $^{13}$C-NMR, see Table 1; ESI-MS (positive mode) $m/z$ 675.3 [M+Na]$^+$; HR-ESI-MS $m/z$ 675.4243 [M+Na]$^+$ (Calcd for C$_{40}$H$_{60}$O$_7$Na, 675.4237).

Cell Culture and Treatments  PANC-1 human pancreatic adenocarcinoma cells were obtained from the Korea Cell Line Bank (KCLB). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) medium (Grand Island, NY, U.S.A.) supplemented with 10% heat-incubated fetal bovine serum (FBS; Hyclone), penicillin (100 U/mL), streptomycin (100 µg/mL), and 3.7 mg/mL NaHCO$_3$. PANC-1 cells were cultured at 37°C in humidified atmosphere containing 5% CO$_2$. For glucose deprivation condition, 2-deoxyglucose (2DG, 100 µM) was added to each well (20 mM), and the plates were re-incubated. After incubation for 24 h, MTT reagent (5 mg/mL) was added to each of the wells, and the plate was incubated for an additional 50 min at 37°C. The media were then removed, and the intracellular formazan product was dissolved in 100 µL of DMSO. The absorbency of each well was then measured at 540 nm using the enzyme-linked immunosorbent assay (ELISA) reader (BioRad, Model 680, U.S.A.), and the percentage viability was calculated.

Conflict of Interest  The authors declare no conflict of interest.

Supplementary Materials  The online version of this article contains supplementary materials. Spectroscopic data of compounds 1 and 2 are available as supplementary material.

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