Two New Oleanane-type Triterpenoids from Methanolyzed Saponins of Momordica cochinonensis

Rong Fan, a,b Rong-Rong Cheng, a Hong-Tao Zhu, a Dong Wang, a Chong-Ren Yang, a Min Xu, a* and Ying-Jun Zhang a,b

a State Key Laboratory of Phytochemistry and Plant Resources of West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, P. R. China
b Yunnan University of Traditional Chinese Medicine, Kunming, 650500, P. R. China

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Two new oleanane-type triterpenoid saponins (1 and 2) were isolated from the methanolyzed total saponins of the seeds of Momordica cochinonensis (Lour.) Spr. together with 16 known compounds (3–18). Their structures were elucidated on the basis of detailed spectroscopic, including 1D and 2D NMR, mass spectrometric, methanolyis and LC-MS analysis. All the isolates were tested for their cytotoxic activities against five human cancer cell lines (HL-60, SMMC-7721, MDMB-231, A-549, and SW-480) and the glucose uptake activity. The known compound 6 exhibited toxic effects against HL-60 with an IC50 value of 18.1 μM, while 10 showed cytotoxicity against SMMC-7721 and A-549 cell lines, with IC50 values of 34.4 and 32.8 μM, respectively. In addition, the new compound 2 showed glucose uptake activity with a glucose consumption value of 0.29 μM at 10 μM concentration.

Keywords: Momordica cochinonensis, Oleanane-type triterpenoid saponins, Cytotoxic activity, Glucose uptake activity.

Momordica cochinonensis (Lour.) Spr. (Cucurbitaceae) is a perennial climber widely cultivated in southern China and southeastern Asia. The seeds having been used to treat pyocutaneous diseases [1], is officially validated as Mubiez and listed in the Chinese Pharmacopoeia, while the roots have been used in China for their expectorant and antiphlogistic activities [2].

Previously, several new saponins, e.g. momordica saponins I and II from the seeds, and momordicasaimins I, II and III from the roots, were reported from M. cochinonensis. Momordica saponins as the main saponins in the titrated plant were reported to have various biological activities, such as antitumor, antioxidant, and hypoglycemic effects [3–5]. As a continuing study on active saponins, 18 oleanane-type triterpenoid glycosides, including two new ones, 3-O-β-D-glucuronosyl-6,3-lactone-gypsogenin (1) and 3-O-α-L-rhamnopyranosyl-(1→3)-6′-O-methyl-β-D-glucuronopyranosyl-gypsogenin (2) were isolated from the methanolyzed total saponins of the seeds of M. cochinonensis.

Their structures were determined on the basis of spectroscopic, methanolyis and LC-MS analyses. Moreover, all the isolated compounds were tested for their cytotoxic activities against five human cancer cell lines (HL-60, SMMC-7721, MDMB-231, A-549, and SW-480) and the glucose uptake activity.

The MeOH extract of the defatted seeds of M. cochinonensis was suspended in water and partitioned successively with light petroleum, CHCl3, and n-BuOH. The aqueous layer was fractionated by D101 column chromatography (CC), eluting with H2O to remove saccharides and then MeOH to give the total saponin, which was subjected to repeated CC over silica gel, RP-18, and MCI-gel HP20P, after methanolyis, to afford two new oleanane-type triterpenoid saponins 1 and 2, together with 16 known saponins (3–18). The known compounds were identified as 3-O-6′-O-methyl-β-D-glucuronopyranosyl-gypsogenin (3) [1], arjunolic acid (3) [6], gypsogenic acid (5) [7], 3-O-6′-O-methyl-β-D-glucuronopyranosyl-gypsogenin (6), 3-O-β-D-glucuronopyranosylgypsogenin (7) [1], oleragenin (8) [8], oleaginac (9) [1], oleanolic acid (10) [7], hederagenin (11) [9], 3-O-6′-O-methyl-β-D-glucuronopyranosyl-gypsogenin (12) [1], 3-O-β-D-galactopyranosyl-(1→2)-6′-O-methyl-β-D-glucuronopyranosyl-gypsogenin (13) [1], 3-O-β-D-galactopyranosyl-(1→2)-6′-O-methyl-β-D-glucuronopyranosyl-gypsogenin (14) [1], 3-O-β-D-galactopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)-β-D-glucuronopyranosyl-gypsogenin (15) [10], 3-O-β-D-galactopyranosyl-(1→2)-α-L-rhamnopyranosyl (1→3)-6′-O-methyl-β-D-glucuronopyranosyl-gypsogenin (16) [1], 3-O-β-D-galactopyranosyl-(1→2)-6′-O-methyl-β-D-glucuronopyranosyl-gypsogenin (17) [1], and 3-O-β-D-galactopyranosyl-(1→2)-α-L-rhamnopyranosyl (1→3)-6′-O-methyl-β-D-glucuronopyranosyl-gypsogenin (18) [1].

Figure 1: Structures of 1-18 isolated from M. cochinonensis.
pyranosyl-quilliac acid (18) [1], by comparing with authentic samples and their spectroscopic and physical data with literature values.

Compound 1 was obtained as a white amorphous powder. Its molecular formula was assigned to be C_{30}H_{40}O_{12} on the basis of the positive ion HR-ESI-ES (m/z 651.3510 [M+Na]^+) as calcd. for C_{30}H_{40}O_{12}Na, 651.3504) and 13C NMR (DEPT) spectra. The IR absorptions at 3442, 1783, 1720, 1695, and 1631 cm\(^{-1}\) indicated the presence of hydroxyl, double bond and carbonyl units. In the 13C NMR (DEPT) spectra, apart from signals of one hexosyl moiety (anomeric carbon at \(\delta_C \approx 111.2\)), the other 30 carbon signals arose from six methylenes (\(\delta_C \approx 26.0, 23.7, 23.5, 23.3, 23.2, 23.1\), 31, 32, 33, 34, and 35), five methylenes (\(\delta_C \approx 26.0, 23.7, 23.5, 23.3, 23.2, 23.1\), 31, 32, 33, 34, and 35), six methylenes, including one oxygen-bearing (\(\delta_C \approx 80.0\)) and one olefinic (\(\delta_C \approx 123.2\)) methyne, nine quaternary carbons, including one olefinic (\(\delta_C \approx 144.8\)) and two carbonyl (\(\delta_C \approx 207.1\) and 180.1) carbons. In the 1H NMR spectrum, six methyl singlets (\(\delta_H \approx 0.71, 0.90, 0.96, 1.09, 1.23,\) and 1.23), one olefinic proton at \(\delta_H \approx 5.41\) (m) and one aldehyde group proton at \(\delta_H \approx 9.58\) (s) were observed, in addition to one anomeric proton (\(\delta_H \approx 5.65\), brs). The aforementioned NMR data were closely related to those of compound 3, a known gysopogenin glycoside isolated from the title plant [1], except for the signals belonging to the sugar moiety. The 1H-1H COSY and HMBC data further confirmed the aglycon of 1 as the same as that of 3. Furthermore, the 1H-1H COSY correlations of \(\delta_H \approx 5.41\) (br d, \(J = 4.0\) Hz, H-4') with \(\delta_H \approx 5.02\) (br d, \(J = 4.0\) Hz, H-5) and \(\delta_H \approx 5.19\) (br d, \(J = 4.0\) Hz, H-3'), as well as the HMBC correlations of H-3' (\(\delta_H \approx 5.20\)), H-4' (\(\delta_H \approx 5.41\)) and H-5' (\(\delta_H \approx 5.02\)) with the carbonyl carbon (\(\delta_C \approx 175.7\), C-6') revealed that the sugar moiety of 1 should be \(\beta\)-D-glucurono-6,3-lactone [11]. This sugar unit was reported to be easily converted from the 6′-O-\(\beta\)-methyl-glucurono-moiety under acid conditions [11]. In order to confirm further the conversion, a MeOH (5 mL) and HSO\(_4\) (96%, 25 \(\mu\)L) solution of compound 3 was agitated under 85°C. After 3 hours, the reaction mixture was checked by TLC and HPLC analyses, from which compound 1 was detected and further confirmed by the LC-MS analysis (Supplementary data, Figure S9). The result supported that the sugar moiety of 1 was 6′-D-glucurofuranosidurono-6,3-lactone, which was located at C-3 of the aglycon on the basis of the HMBC correlation between H-1′ and C-3. Therefore, the structure of 1 was assigned as 3-O-\(\beta\)-D-glucurofuranosidurono-6,3-lactone-gysopogenin.

Compound 2 was isolated as white amorphous powder. The molecular formula was assigned as C_{30}H_{40}O_{12} by HRESIMS (m/z 829.4354 [M+Na]^+) as calcd. for C_{30}H_{40}O_{12}Na, 829.4345) and 13C NMR (DEPT) spectra. The 1H and 13C NMR data of 2 were similar to those of 3-O-6′-O-methyl-\(\beta\)-D-glucuronopyranosyl-gysopogenin (3), except for the appearance of an additional hexosyl moiety, which was assigned as \(\alpha\)-rhamnopyranosyl (\(\delta_H \approx 103.0, 69.9, 74.1, 71.2, 72.5, 18.6\)), by comparison of its 1H and 13C NMR data with literature values [1,12]. Acid hydrolysis of 2 afforded L-rhamnose. The downfield shift of C-3′ of the 6′-O-\(\beta\)-methyl-\(\beta\)-D-glucuronosyl moiety in 2 to \(\delta_C \approx 81.8\) indicated that the \(\alpha\)-L-rhamnopyranosyl unit was located on C-3′. This was confirmed by the HMBC correlation between H-1′ (\(\delta_H \approx 6.26\)) and C-3′ (\(\delta_C \approx 81.8\)). All the proton and carbon signals from the two sugar moieties could be assigned unambiguously by HSQC-TOCSY experiment. Thus, the structure of 2 was determined to be 3-O-\(\alpha\)-L-rhamnopyranosyl(1→3)-6′-O-methyl-\(\beta\)-D-glucuronopyranosyl-gysopogenin.

All the isolates 1-18 were evaluated for their cytotoxicities against five human cancer cell lines, myeloid leukemia (HL-60), hepatocellular carcinoma (SMMC-7721), pancreatic cancer (PANC-1), lung cancer (A-549), and colon cancer (SW-480), using the MIT method as reported previously, with DDP and taxol as positive controls. The known saponin 6 exhibited a toxic effect against HL-60 with an IC_{50} value of 18.1 \(\mu\)M, while 10 showed cytotoxicity to SMMC-7721 and A-549 cell lines, with IC_{50} values of 34.4 and 32.8 \(\mu\)M, respectively (Table 2). Moreover, 1-18 were subjected to glucose uptake assay. Only compound 2 showed glucose uptake activity with a glucose consumption value of 0.29 \(\mu\)M at a concentration of 10 \(\mu\)M (Table 3).

In summary, two new oleane-type triterpenoid saponins, 3-O-\(\beta\)-D-glucurofuranosidurono-6,3-lactone-gysopogenin (1) and 3-O-\(\alpha\)-L-rhamnopyranosyl(1→3)-6′-O-methyl-\(\beta\)-D-glucuronopyranosyl-gysopogenin (2), were isolated from the seeds of M. coelichinensis, in addition to 16 known saponins. The known saponins 6 and 9 exhibited weak cytotoxicity against some cancer cells, while 2 showed weak glucose uptake activity.
Saponins from methanolized saponins of *Momordica cochinchinensis*

<table>
<thead>
<tr>
<th>Compound</th>
<th>HL-60</th>
<th>SMMC-7721</th>
<th>A-549</th>
<th>MCF-7</th>
<th>SW480</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.1</td>
<td>6.7</td>
<td>8.4</td>
<td>13.5</td>
<td>17.3</td>
</tr>
<tr>
<td>9</td>
<td>&gt;40</td>
<td>34.4</td>
<td>32.8</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>DDEP</td>
<td>1.1</td>
<td>5.7</td>
<td>8.4</td>
<td>13.5</td>
<td>17.3</td>
</tr>
<tr>
<td>Taxol</td>
<td>&gt;0.008</td>
<td>&gt;0.008</td>
<td>&gt;0.008</td>
<td>&gt;0.008</td>
<td>&gt;0.008</td>
</tr>
</tbody>
</table>

Table 3: The glucose consumption of compound 2 (μM).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Well 1</th>
<th>Well 2</th>
<th>Well 3</th>
<th>Average</th>
<th>Standard Deviation</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (10 μM)</td>
<td>0.27</td>
<td>0.26</td>
<td>0.25</td>
<td>0.26</td>
<td>0.011</td>
<td>0.0044</td>
</tr>
<tr>
<td>Insulin (100 μM)</td>
<td>0.51</td>
<td>0.47</td>
<td>0.45</td>
<td>0.47</td>
<td>0.031</td>
<td>0.0004</td>
</tr>
<tr>
<td>Rosiglitazone (100 μM)</td>
<td>0.32</td>
<td>0.29</td>
<td>0.35</td>
<td>0.32</td>
<td>0.006</td>
<td>0.0185</td>
</tr>
<tr>
<td>2</td>
<td>0.30</td>
<td>0.30</td>
<td>0.28</td>
<td>0.29</td>
<td>0.007</td>
<td>0.0100</td>
</tr>
</tbody>
</table>

General: Optical rotations were measured on a P-1020 Polari meter (JASCO, Tokyo, Japan) and IR spectra on a Bio-Rad FTS-135 series spectrometer. ¹H, ¹³C NMR, HMOC, and HMQC spectra, in CDCl₃, were recorded with either Bruker DRX-500 or Varian-600 spectrometers operating at 400 or 600 MHz for ¹H NMR and 100 or 150 MHz for ¹³C NMR, respectively. Coupling constants are expressed in Hz, and chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. ESI-MS and HR-ESI-MS were recorded on VG Auto Spec-300 and API QSTAR Pulsar-1 spectrometers, respectively. Column chromatography (CC) was done on macro porous resin D101 (Shandong Lukang Pharmaceutical Co., Ltd., China), MCI-gel CHP20P (75-150 μm, Mitsubishi Chemical Co., Ltd., Japan), silica gel (200-300 mesh) (Qingdao Marine Chemical and Industrial Factory, China), and RP-18 gel (40-60 μm) (Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed on precoated kieselgel 60 F254 plates, 0.2 mm thick (Merck) with CHCl₃-MeOH-H₂O (9:1:0.1 or 8:2:0.2, v/v) and compounds were detected by spraying with 10% sulfuric acid solution followed by heating.

Plant material: Air-dried seeds of *M. cochinchinensis* were collected from Yunnan Province, China, in July 2013 and authenticated by one of the co-authors (Chong-Ren Yang). A voucher specimen (KIB-Z-00335) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation: Coarsely crushed seeds (50 kg) were defatted with light petroleum (50 L) at room temperature, and then refluxed at 80°C with MeOH (100 L) twice, each time for 3 h. The MeOH extract (2.0 kg) was suspended in H₂O and partitioned successively with light petroleum, CHCl₃, and n-ButOH. The aqueous layer (1.2 kg) was fractionated by D101 CC, eluting with H₂O to remove saccharides, and then MeOH to give total saponins (900 g), which were dissolved in 2N HCl in MeOH (9 L) and refluxed in a water bath for 2 h. The reaction mixtures were subjected to D101 CC, eluting with H₂O, and then MeOH to give methanolized saponins (800 g).

The methanolized saponins (800 g) were subjected to silica gel CC, eluting with CHCl₃-MeOH (99:5 - 7:3, v/v) to afford 5 fractions (Fr. A-E). Fr. A (71 g) was chromatographed over silica gel (CHCl₃-MeOH, 9:1, v/v) to give compound 9 (11 g) and another eight fractions (Fr. A₁ - Fr. A₈). Fr. A₉ (5.8 g) was subjected to MCI-gel CHP20P CC, eluting with a gradient step of MeOH-H₂O (70%-90% MeOH), followed by re-crystallization in MeOH to afford 10 (224 mg). Fr. A₁₀ (7.1 g) was separated on a MCI-gel CH20P column, eluting with aqueous MeOH (70% - 90%) to give 11 (40 mg). Fr. B (5 g) was subjected to RP-18 CC, eluting with MeOH-H₂O (70%-90%) to give 7 fractions (Fr. B₁ - Fr. B₇). Fr. B₈ (600 mg) was chromatographed on silica gel, eluting with CHCl₃-MeOH (9:1, v/v) to yield 8 (108 mg). Fr. B₉ (199 mg) was subjected to silica gel (CHCl₃-MeOH, 9:1, v/v) and RP-18 (MeOH-H₂O, 70%-90%) CC to obtain 6 (61 mg) and 7 (23 mg). Fr. C (10 g) was chromatographed by silica gel CC (CHCl₃-MeOH, 9:1, v/v), followed by re-crystallization in MeOH to give 3 (10 g).

Fr. D (165 g) and E (89 g) were separately subjected to silica gel CC (CHCl₃-MeOH, 8:2, v/v) to obtain 6 (Fr. D₁ - Fr. D₅) and 9 (Fr. E₁ - Fr. E₅) fractions, respectively. Fr. D₆ (640 mg) was purified by MCI-gel CH20P CC (MeOH-H₂O, 70%-90%) to afford 4 (56 mg) and 3 fractions (Fr. D₇₁ - Fr. D₇₃). Fr. D₇₁-D₇₃ (388 mg) was further subjected to silica gel CC (CHCl₃-MeOH, 9:1, v/v) to give 1 (19 mg) and 5 (31 mg). Fr. E₆ (230 mg) was subjected to MCI-gel CH20P CC, eluting with MeOH-H₂O (70%-90%) to give 12 (91 mg). Fr. E₇ (5 g) was applied to MCI-gel CH20P CC (MeOH-H₂O, 70%-90%) to give 3 fractions (Fr. E₇₁ - Fr. E₇₃). Fr. E₇₃ (2.5 g) was chromatographed on a RP-18 column, eluting with a gradient of MeOH-H₂O (from 70% to 90% MeOH) to obtain 2 (111 mg). Fr. E₈ (16.7 g) was subjected to CC on MCI-gel CH20P (MeOH-H₂O, 70%-90%) and RP-18 (MeOH-H₂O, 70%-90%) to afford 13 (2.3 g) and 14 (95 mg). Fr. E₁₃ (13.7 g) was separated through RP-18 (MeOH-H₂O, 70%-90%), MCI-gel CH20P (MeOH-H₂O, 70%-90%) to give 15 (25 mg), 16 (2 g) and 17 (91 mg). Fr. E₁₆ (10 g) was subjected to silica gel CC, eluting with CHCl₃-MeOH (7:3, v/v), to give 18 (577 mg).

3-O-β-D-Glucosidurosinoduro-6,3-lactone-gypsogenin (1)

White amorphous powder. [α]D ° = -3.13 (c 0.1, MeOH). IR (KBr): 3442, 2931, 1783, 1720, 1631, 1454, 1385, 1071 cm⁻¹. ¹H and ¹³C NMR: Table 1. ESI-MS: m/z 651 [M + Na]⁺.

HR-ESI-MS (positive): m/z 651.3510 [M+Na⁺] (calcd for C₂₃H₂₃O₁₂Na, 651.3504).

3-O-α-L-Rhamnopyranosyl-(1→3)-6′-O-methyl-β-D-gluconopyrano- pyranosyl-gypsogenin (2)

White amorphous powder. [α]D ° = -13.06 (c 0.1, MeOH). IR (KBr): 3439, 2946, 1747, 1722, 1633, 1450, 1387, 1048 cm⁻¹. ¹H and ¹³C NMR: Table 1. ESI-MS: m/z 805 [M+H]⁺.

HR-ESI-MS (positive): m/z 829.4354 [M+Na⁺] (calcd for C₂₃H₂₃O₁₂Na, 829.4345).

Methanalysis of compound 3: A mixture of compound 3 (15 mg), MeOH (5 mL) and H₂SO₄ (96%, 25 μL) in a 10 mL reaction vial was kept at 85°C for 3 h with agitation [11]. The reaction mixture was subjected directly to LC-MS analysis to confirm the production of compound 1 (m/z 651 [M + Na]⁺). Analytical high pressure liquid chromatography (HPLC) was performed on a SunFire C₁₈, 5 μm column (250 × 4.6 mm) with gradient elution from 30% to 80% aqueous CH₃CN in flowing rate of 1 mL/min within 60 min, with detection between 210 and 400 nm. MS analysis was performed on an ion-trap mass spectrometer with an electrospray interface (ESI), operating in full scan MS mode from 150 to 1500 amu. Samples were analyzed using both negative and positive ionization modes. ESI-MS parameters were as follows: the potential of ESI source, 4 kV; capillary temperature, 400°C.

Acid hydrolysis of compound 2: Compound 2 (20 mg) was hydrolyzed with 2 N HCl/dioxane (1:1, 6 mL) at 60°C under reflux
for 3 h. The reaction mixture was extracted with CHCl₃ 3 times (5 mL × 3). The aqueous layer was neutralized with 2 N NaOH and then dried to obtain a monosaccharide mixture. A solution of the sugar mixture in pyridine (2 mL) was added to L-cysteine methyl ester hydrochloride (1.5 mg) and kept at 60°C for 1 h. Then trimethylsilylimidazole (1.5 mL) was added to the reaction mixture and kept at 60°C for 30 min. The mixture was subjected to GC analysis, run on a Shimadzu GC-14C gas chromatograph equipped with a H₂ flame ionization detector, and 300C2/AC-5 quartz capillary column (30 m × 0.32 mm i.d.). Column temperature: 180-280°C; programmed increase: 3°C/min; carrier gas: N₂ (1 mL/min); detector and injector temperature: 250°C; detector and injector volume: 4 μL; split ratio: 1/50. The configuration of the sugar moiety was determined by comparing the retention time (tR) of each peak with that of the authentic samples. The configuration of the sugar moiety from compound 2 was determined to be L-rhamnose (tR = 18.473 min).

Cytotoxic assay: The cytotoxicities of compounds 1-18 against HL-60, SMMC-7721, A-549, MCF-7 and SW-480 cell lines were assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method [13]. Cells were plated in 96-well plates for 12 h before treatment and continuously exposed to different concentrations of compounds. After 48 h, 20 μL of MTT solution was added to each well, which was incubated for a further 4 h. Then 20% SDS (100 μL) was added to each well. After 12 h at room temperature, the OD value of each well was recorded at 490 nm. The IC₅₀ value of each compound was calculated by the Reed and Muench method. DDP and taxol were used as positive controls.

Glucose uptake assay: The assay for glucose uptake was conducted with minor modifications [14], and 3T3-L1 fibroblasts (ATCC, USA) were cultured and differentiated into adipocytes as reported [15]. Differentiated adipocytes were plated into 96-well plates and pre-incubated with DMEM / HIGH GLUCOSE containing 10% FBS and 1% P/S overnight, and were further incubated with either 10 μM compounds (1-18) or 100 nM Rosi in DMEM / LOW GLUCOSE containing 10% FBS, 1% P/S and 0.2% BSA for 24 h. The medium was collected and its glucose concentrations were determined by the glucose oxidase method using a Glucose Kit. The amount of glucose uptake was calculated by the glucose concentrations of blank wells subtracting the remaining glucose in the cell-plated wells.

Supplementary data: Copies of the spectral data of compounds 1 and 2 are available.

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References