Novel Triterpenoid Alkaloids With Their Potential Cytotoxic Activity From the Roots of Siraitia grosvenorii

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Four novel triterpenoid alkaloids, siragrosvenins A–D (1–4), and two new cucurbitane-type triterpenoids, siragrosvenins E–F (5, 6), together with eight known analogs (7–14), were isolated from the roots of *Siraitia grosvenorii*. Compounds 1–4 possessed a rare cucurbitane-type triterpenoid scaffold, featuring an extra pyrazine unit via the Strecker reaction in the cucurbitane framework. Compound 5 displayed a 6/6/6/5/6/5-fused polycyclic ring system, with an uncommon fused furan and pyran ring in the side chain. All the structures were characterized by extensive spectroscopic analysis, including HRESIMS, NMR, and X-ray crystallographic data. It is worth noting that the DP4+ analysis method was applied for the first time to determine the absolute configurations of the trihydroxybutyl moiety in the side chain of compounds 1–4.

In vitro cytotoxicity screening found that compounds 4, 8, 9, 13, and 14 exhibited remarkable cytotoxic activities against three cell lines with IC50 values ranging from 1.44 to 9.99 μM. Siragrosvenin D shows remarkable cytotoxic activity on MCF-7 cells. As a result, it inhibited the proliferation of MCF-7 cells and reduced their viability via the induction of G2/M phase arrest and significantly induced apoptosis in MCF-7 cells.

Keywords: Cucurbitaceae, *Siraitia grosvenorii*, cucurbitane-type triterpenoid, pyrazine, cytotoxicity

INTRODUCTION

Triterpenoids are commonly distributed in higher plants and have attracted much attention due to their structural diversities and broad range of bioactivities (Chen et al., 2005). Cucurbitane triterpenoids, as an important part of the triterpenoid family, are famous for their highly oxygenated skeletons, which are obtained initially from the Cucurbitaceae genus. More importantly, members of this group of natural products have been reported for their diverse pharmacological effects, such as anticancer (Garg et al., 2018), anti-inflammatory (Liu et al., 2020), antihyperglycemic (Sun et al., 2018), and antilipidemic activities (Huang et al., 2012; Cai et al., 2015). In addition, cucurbitane triterpenoids possessing a nitrogen-containing heterocycle are rarely reported. The insertion of heteroatoms can often improve the biological activity of chemicals, which has attracted extensive attention of scholars (Pettit et al., 1988; Urban et al., 2007).

*Siraitia grosvenorii* Swingle (Cucurbitaceae) is a perennial plant growing in the southern part of China, Guangxi province. The roots of *S. grosvenorii* are traditionally used to treat tongue fat, meningitis sequelae, diarrhea, and rheumatoid arthritis as a folk medicine in China (Qing et al.,...
2017). However, few investigations were conducted on the isolation and identification of compounds presented in the roots. In order to search for active natural products from this plant, we isolated four uncommon triterpenoid alkaloids (1–4), two new cucurbitane-type triterpenoids (5, 6) and eight known compounds (7–14) (Figure 1). Among them, compounds 1–4 contained a novel cucurbitane-type triterpenoid skeleton with an additional pyrazine unit via a carbon–nitrogen linkage in the structure. Compound 5 showed an unexpected triterpenoid structure with a 6/6/6/5/6/5-fused polycyclic ring system, through aldol condensation. The cytotoxicity of these compounds was evaluated against three human cancer cell lines (MGC-803, MCF-7, and CNE-1) in vitro. Herein, we presented the isolation, structure elucidation, and cytotoxicity of these cucurbitane-type triterpenoids. Furthermore, we also conducted a preliminary investigation on the effects of siragrosvenin D, which could arrest the cell cycle and significantly induce apoptosis in MCF-7 cells.

**MATERIALS AND METHODS**

**Plant Material**

The roots of *S. grosvenorii* were collected from the Yongfu county, Guangxi province and identified by Prof. Ma Xiaojun (Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College) in October 2019. The voucher specimen (accession number 2019004) was stored at the herbarium of our institute.

**General Experimental Procedures**

UV spectra data were recorded on a Thermo Scientific Genesys 10S spectrophotometer (Thermo Scientific, Madison, WI, United States). Infrared spectra were obtained on a Nicolet iS5 FT-IR spectrophotometer (Thermo Scientific, Madison, WI, United States). HRESIMS data were measured on a Thermo Scientific LTQ-Orbitrap XL (Bremen, Germany). Optical rotations were measured with an Anton Paar MCP 200 automatic polarimeter (Anton Paar GmbH, Graz, Austria) in MeOH at 25°C. 1D and 2D NMR spectra data were recorded on a Bruker AV III 600 NMR spectrometer (Rheinstetten, Germany). Column chromatography was performed by using silica gel (100–200, 300–400 mesh, Qingdao Haiyang Chemical Co., Ltd., Qingdao, China) and Sephadex LH-20 (Pharmacia Biotech, Sweden). Thin-layer chromatography (TLC) was performed on precoated silica gel GF254 plates (0.25 mm, Qingdao Haiyang Chemical Co., Ltd., Qingdao, China). Semi-preparative HPLC was carried out on a LC-UV system (Waters 2,549, United States) with a YMC-C18 column (250 × 10 mm, 5 μm, Japan) and Agilent SB-Phenyl (250 × 10 mm, 5 μm, United States), detected by a binary channel UV detector at 210 and 254 nm. All solvents used for HPLC were of HPLC grade obtained from Thermo Fisher.

**Extraction and Isolation**

The air-dried roots (20.0 kg) of *S. grosvenorii* were extracted three times by 90% ethanol under reflux. The solvent was then removed
under reduced pressure to yield a crude extract (1,300 g), which
was then suspended in water (3 × 2000 ml) and partitioned with
petroleum ether, dichloromethane, ethyl acetate, and n-butanol.
The CH2Cl2 residue (200 g) was chromatographed on silica gel
CC using an increasing gradient of CH2Cl2-MeOH (150:1 to 0:1,
v/v) to afford seven main fractions (A−G).
Fraction B (30 g) was submitted to a silica gel column by using
CH2Cl2-MeOH (120:1 to 0:1 v/v) as an eluent to generate
fractions B1−B5. Subsequently, further purification of B2 was
separated as four parts over an MCI gel column with a
MeOH-H2O gradient system (7:3–0:1 v/v). B2-1 was purified
by a Sephadex LH-20 column (4 × 120 cm) to yield
compound 7 (10 mg). Compound 1 (3.0 mg, tR 23 min) and
compound 2 (8.0 mg, tR 25 min) were isolated from fraction
B2-3 (24 mg) via semi-preparative HPLC with 40% MeOH at
2 ml/min. B2-4 (33 mg) was chromatographed by semi-
preparative HPLC (CH3CN-H2O, 65:35, v/v) to furnish
compound 3 (3.2 mg, tR 15 min), compound 4 (2.4 mg, tR

### TABLE 1

| No. | 1     | 2     | 3     | 4     | 5     | 6     |
|-----|-------|-------|-------|-------|-------|-------|
| 1   | 3.22, overlap | 3.22, overlap | 3.28, dd (6, 12) | 3.27, dd (6, 12) | 2.50, m | 2.52, m |
| 2   | —     | —     | —     | —     | 4.09, m | 4.12, m |
| 3   | —     | —     | —     | —     | 3.43, m | 3.45, d (9) |
| 4   | —     | —     | —     | —     | —     | —     |
| 5   | —     | —     | —     | —     | —     | —     |
| 6   | 5.92, m | 5.91, m | 5.90, m | 5.89, m | 5.69, m | 5.72, m |
| 7   | 2.36, m | 2.38, m | 2.37, m | 2.37, m | 2.33, m | 2.33, m |
| 8   | 2.01, m | 2.00, m | 1.97, m | 1.98, m | 1.97, m | 2.01, m |
| 9   | —     | —     | —     | —     | —     | —     |
| 10  | 2.83, m | 2.84, m | 2.75, m | 2.75, m | 2.66, m | 2.71, m |
| 11  | —     | —     | —     | —     | —     | —     |
| 12  | 3.22, overlap | 3.20, d (12) | 3.20, d (12) | 3.20, d (12) | 3.16, d (12) | 3.21, d (12) |
| 13  | —     | —     | —     | —     | —     | —     |
| 14  | —     | —     | —     | —     | —     | —     |
| 15  | 1.94, m | 1.98, m | 1.92, m | 1.94, m | 1.95, m | 1.69, m |
| 16  | 1.73, m | 1.76, m | 1.72, m | 1.75, m | 1.65, m | 1.98, m |
| 17  | 4.96, m | 5.10, m | 4.96, m | 5.11, m | 5.00, m | 4.92, m |
| 18  | 2.93, d (7.2) | 3.00, d (7.2) | 2.93, d (7.2) | 2.98, d (7.2) | 2.10, d (12) | 2.18, d (12) |
| 19  | 1.27, s | 1.27, s | 1.27, s | 1.26, s | 1.22, s | 1.20, s |
| 20  | 1.25, s | 1.25, s | 1.24, s | 1.23, s | 1.24, s | 1.27, s |
| 21  | 1.68, s | 1.70, s | 1.64, s | 1.71, s | 1.36, s | 1.49, overlap |
| 22  | —     | —     | —     | —     | 1.94, overlap | 2.70, m |
| 23  | 3.33, m | 7.34, d (15.8) | 3.34, m | 7.35, d (15.8) | —     | 5.15, m |
| 24  | 2.45, m | 7.41, d (15.8) | 2.45, m | 7.41, d (15.8) | 2.33, m | 6.06, m |
| 25  | —     | —     | —     | —     | 1.71, m | —     |
| 26  | 1.50, s | 1.55, s | 1.51, s | 1.55, s | 3.79, m | 4.30, s |
| 27  | —     | —     | —     | —     | 3.74, m | —     |
| 28  | 1.46, s | 1.52, s | 1.48, s | 1.52, s | 4.22, m | 1.79, s |
| 29  | —     | —     | —     | —     | 4.09, m | —     |
| 30  | 1.63, s | 1.68, s | 1.62, s | 1.61, s | 1.47, s | 1.49, overlap |
| 31  | 1.47, s | 1.46, s | 1.43, s | 1.49, s | 1.31, s | 1.32, s |
| 32  | 1.48, s | 1.53, s | 1.49, s | 1.47, s | 1.34, s | 1.39, s |
| 33  | 8.78, s | 8.76, s | —     | —     | —     | —     |
| 34  | 3.62, d (9.8, 14) | —     | 3.79, dd (9.8, 14) | —     | 3.79, dd (9.8, 14) | —     |
| 35  | 4.36, m | 4.36, m | 4.33, m | 4.33, m | —     | —     |
| 36  | 4.83, m | 4.84, m | 4.80, m | 4.80, m | —     | —     |
| 37  | 4.52, m | 4.52, m | 4.52, m | 4.51, m | —     | —     |
| 38  | 4.40, m | 4.40, m | 4.38, m | 4.40, m | —     | —     |
| 39  | —     | —     | —     | —     | —     | —     |
| 40  | 1.90, s | 1.88, s | 1.90, s | 1.88, s | —     | —     |
Wang et al. Novel Compounds From Siragrosvena C (I): pale-yellow powder; (α)D 25 +43 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 205 (1.19) and 282 (0.90) nm; IR νmax 3,419, 2,968, 2,928, 1,697, 1,392, 1,370, 1,260, and 1,205 cm⁻¹; 1H NMR (Pyrdine-D5, 600 MHz) and 13C NMR (Pyrdine-D5, 150 MHz) data are shown in Tables 1, 2; HRESIMS (positive mode) m/z 707.3880 (M + Na)+ (calculated for C38H54N2O9Na, 707.3870).

Saragrosvenin B (2): pale-yellow powder; (α)D 25 +25 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 205 (1.32) and 282 (1.01) nm; IR νmax 3,444, 2,976, 2,942, 1,698, 1,369, 1,354, and 1,222 cm⁻¹; 1H NMR (Pyrdine-D5, 600 MHz) and 13C NMR (Pyrdine-D5, 150 MHz) data are shown in Tables 1, 2; HRESIMS (positive mode) m/z 705.3724 (M + Na)+ (calculated for C36H52N2O9Na, 705.3727).

Saragrosvenin C (3): pale-yellow powder; (α)D 25 +5 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 207 (2.09) and 282 (1.35) nm; IR νmax 3,420, 2,966, 2,928, 1,697, 1,388, 1,371, 1,261, and 1,024 cm⁻¹; 1H NMR (Pyrdine-D5, 600 MHz) and 13C NMR (Pyrdine-D5, 150 MHz) data are shown in Tables 1, 2;
HRESIMS (positive mode) m/z 707.3882 (M + Na)^+ (calculated for C_{38}H_{56}N_{2}O_{9}Na, 707.3883).

Siragrosvenin D (4): pale-yellow powder; (α)_{D}^{25} +9 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 207 (2.35) and 282 (0.90) nm; IR ν_{max} 3,419, 2,967, 2,927, 1,687, 1,372, 1,260, and 1,023 cm^{-1}; 1H NMR (Pyridine-D_{5}, 600 MHz) and 13C NMR (Pyridine-D_{5}, 150 MHz) data are shown in Tables 1, 2; HRESIMS (positive mode) m/z 705.3730 (M + Na)^+ (calculated for C_{38}H_{54}N_{2}O_{9}Na, 705.3727).

Siragrosvenin E (5): white powder; (α)_{D}^{25} +159 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 207 (2.13) nm; IR ν_{max} 3,419, 2,965, 2,882, 1,688, 1,055, and 1,029 cm^{-1}; 1H NMR (600 MHz, Pyridine-D_{5}) and 13C NMR (150 MHz, Pyridine-D_{5}) data are shown in Tables 1, 2; HRESIMS (positive mode) m/z 541.3126 (M + Na)^+ (calculated for C_{30}H_{46}O_{7}Na, 541.3141).

Siragrosvenin F (6): white powder; (α)_{D}^{25} +59 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 228 (2.60) nm; IR ν_{max} 3,391, 2,967, 2,882, 1,687, 1,373, 1,068, and 1,027 cm^{-1}; 1H NMR (600 MHz, Pyridine-D_{5}) and 13C NMR (150 MHz, Pyridine-D_{5}) data are shown in Tables 1, 2; HRESIMS (positive mode) m/z 543.3301 (M + Na)^+ (calculated for C_{30}H_{46}O_{7}Na, 543.3297).

13C NMR Calculations
The 13C NMR spectra were calculated according to the reported methods (Buevich and Elyashberg, 2016). The computational data were fitted in the GraphPad Prism 7. The process is also described in detail in Supplementary Material.

X-Ray Crystallographic Analysis
Colorless needle crystals of compound 5 were crystallized in CH_{3}CN–H_{2}O (5:1) at room temperature. Crystal data: C_{30}H_{46}O_{7}, hexagonal, a = 22.7332 (2), b = 22.7332 (2), c = 9.80650 (10) Å, β = 90°, U = 4,388.81 (9) Å³, T = 99.99 (10), space group P6_3, α = 6, μ (Cu Kα) = 1.54184, and Flack parameter = -0.03 (4). A total of 49,416 reflections were measured, 6,100 unique (R_{int} = 0.0394) which were used in all calculations. The final R1 was 0.0331 (I > 2σ (I)), and the wR_{2} was 0.0916 (all data). Crystal size: 0.32 × 0.06 × 0.05 mm³.

The crystallographic data have been deposited in the Cambridge Crystallographic Data Center (CCDC), and the CCDC deposition number is CCDC 2151134. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html.

Cell Viability Assays
The cellular viability of compounds 1–14 was evaluated using the MTT procedure with MGC-803, MCF-7, and CNE-1 cancer cell lines. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and cultured at a density of 3 × 10^4 cells/mL in a 96-well microtiter plate. After 24 h of incubation, six various concentrations of each agent dissolved in dimethyl sulfoxide (DMSO) were then added in the wells. Each concentration of each agent dissolved in dimethyl sulfoxide (DMSO) were then added in the wells. Each concentration of each agent dissolved in dimethyl sulfoxide (DMSO) were then added in the wells. Each concentration of each agent dissolved in dimethyl sulfoxide (DMSO) were then added in the wells. Each concentration of each agent dissolved in dimethyl sulfoxide (DMSO) were then added in the wells.

After shaking for 5 min, the absorbance was measured with a microplate reader at 570 nm (SpectraFluor, TECAN, Sunrise, Austria).

Cell Cycle Analysis
The MCF-7 cells were plated in a six-well plate at a density of 3 × 10^4 cells per well and treated with compound 4 (0, 5, 10, and 20 μM). After 24 h, the cells were fixed in ice-cold ethanol (70%) at 4°C overnight. After the cells were suspended in 0.1% Triton X-100 and 100 μg/ml RNase A, 5 μL PI solution was added and incubated for 30 min (Ueno et al., 2021). Then, the sample was analyzed by a flow cytometry FACS Verse. Modifit LT 4.0 was used to analyze the obtained data.

Apoptosis Analysis
The MCF-7 cells were pre-treated with compound 4 (0, 5, 10, and 20 μM) for 24 h. After washing with PBS, the cells were incubated with 5 μL annexin V in binding buffer for 30 min at room temperature in the dark, followed by 10 μL PI for 5 min. FACS Calibur flow cytometry (Becton Dickinson, United States) was used to detect and analyze the stained cells. The apoptosis rate was reported as the percentage of apoptotic cells to the total number of cells.

Colony Formation Assay
The MCF-7 cells were plated in a six-well plate at a density of 250 cells per well and were treated with different concentrations of compound 4 (0, 0.1, 0.5, 1, 2, and 4 μM) for 14 days. The drugs were removed, and cells were washed twice with PBS. Then, the cells were fixed in methanol for 10 min and stained with 0.1% crystal violet solution for 30 min at room temperature. Finally, PBS was used to wash the cells to visualize the colonies (Ni et al., 2018).

Cell Morphology Observation and AO/EB Staining Assay
The MCF-7 cells were seeded at a density of 2 × 10^4 cells/well onto 24-well plates and were treated with different concentrations of compound 4 (0, 5, 10, and 20 μM) for 24 h. After discarding the cell culture medium, some cells were added with 500 μL AO/EB staining solution for 5 min in the dark. Subsequently, photographs were taken under a fluorescence microscope.

Statistical Analysis
All data were analyzed by GraphPad Prism version 5.0 and were presented as the mean ± SD in at least three independent experiments. Student’s t-test and one way ANOVA were conducted to evaluate significant distinctions. Values of p < 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION
Compound Structure Elucidation
Compound 1 was isolated as a pale-yellow powder. Its molecular formula C_{38}H_{56}N_{2}O_{9}Na was indicated by its HRESIMS at m/z 707.3880 (M + Na)^+, calculated for C_{38}H_{56}N_{2}O_{9}Na 707.3883,
suggesting 12 degrees of unsaturation. The absorption bands at 3,419 and 1,697 cm\(^{-1}\) in the IR spectrum suggested the existence of the hydroxyl and carbonyl groups in compound 1. The characteristic absorption bands at 205 and 280 nm in the UV spectrum provided evidence for the existence of the aromatic group. The \(^1\)H NMR spectrum (Table 1) displayed signals attributed to nine methyl groups at \(\delta_H\) 1.25, 1.27, 1.46, 1.47, 1.48, 1.50, 1.63, 1.68, and 1.90 (each 3H, s); a set of oxygenated proton signals at \(\delta_H\) 4.96 (1H, t), 4.83 (1H, t), 4.52 (1H, m), 4.40 (1H, m), and 4.36 (1H, m) suggested its polyhydroxy property. One olefinic proton at \(\delta_H\) 5.92 (1H, m) indicated the presence of double bonds, and one downfield proton at \(\delta_H\) 8.78 (1H, s) implied the heterocyclic aromatic ring. The \(^13\)C-APT NMR spectrum (Table 1) revealed 38 carbon resonances, including two keto carbonyls at \(\delta_C\) 213.7 and 215.4, one ester carbonyl at \(\delta_C\) 170.5, and six olefinic carbons and aromatic carbons at \(\delta_C\) 155.7, 153.8, 151.4, 144.2, 142.1, and 121.0. The rest of the signals were ascribed to nine methyls, eight methylenes (including one oxygenated carbon at \(\delta_C\) 65.5), five methines (including three oxygenated carbons at \(\delta_C\) 76.8, 73.5, and 70.7), and six quaternary carbons (including two oxygenated carbons at \(\delta_C\) 80.5 and 81.9). All protons are assigned to their corresponding carbons with the help of the HSQC spectrum. In fact, the results after the comparison of these data with the known ones suggested that compound 1 showed the same B–C–D ring and the C-17 side chain structure as 23,24-dihydrocucurbitacin E (Wu et al., 2004), except for the additional four aromatic carbons (\(\delta_C\) 155.7, 153.8, 151.4, and 144.2) and a trihydroxybutyl side chain. In the HMBC spectrum, the correlations from \(\delta_H\) 8.78 (1H, s) to C-2 (\(\delta_C\) 151.4) and C-33 (\(\delta_C\) 153.8) implied that the four downfield aromatic carbons formed a closed-loop system. Considering the two N atoms in the molecular formula, an extra pyrazine unit was established in the structure (Seeman et al., 1992). The HMBC correlations (Figure 1) from H-1, H-28, H-29 to C-3 (\(\delta_C\) 155.7) and C-5 and from H-1 to C-2 (\(\delta_C\) 151.4) revealed that the pyrazine ring was adjacent to ring A. The presence of 36,37,38-trihydroxybutyl moiety was established by the \(^1\)H–\(^1\)H COZY (Figure 2) correlations of H-36/H-37/H-38 and the key HMBC correlations of H-35 to C-36 and C-37, H-36 to C-35 and C-37, and H-38 to C-36 and C-37 (Wang et al., 2014). Subsequently, the trihydroxybutyl moiety was confirmed to attach to C-33 from the HMBC correlations of H-35 to C-33, C-32, and C-3 and H-32 to C-1, C-2, and C-3. Thus, the planar structure of 1 was established unambiguously.

The relative configuration of 1 was evaluated by its NOESY spectrum (recorded in Pyridine-d5). The NOESY correlations (Figure 2) from H-19 to H-18, H-19 to H-8, H-30 to H-10, H-30 to H-17, and H-18 to H-16 revealed that H-18, H-19, H-8, and H-16 are β-oriented, H-30 and H-10, and H-17 are α-oriented. We tried to determine the absolute configuration of compound 1 by crystallization but failed. However, the clear comparison of NMR data with cucurbitacin E, whose absolute configuration has been fully determined, indicated that the cucurbitane partial structure at C-8, C-9, C-10, C-13, C-14, C-16, and C-17 of compound 1 was S, R, R, S, R, and R, respectively (Wu et al., 2004), which was completely consistent with the biogenic pathway of cucurbitane triterpenes. The relative configuration of the 36,37,38-trihydroxybutyl moiety was determined by J-based NMR. The low-temperature NMR (–4°C, CD3OD) of 1 revealed a large coupling constant between H-36 and H-37 (J = 9.2 Hz) (Wang et al., 2014), indicating an antirelationship between the two protons (Li et al., 2015). In addition, a significant NOESY correlation of H-37/H-35/H-38/H-36 indicated a threo configuration (Figure 3) of H-36 and H-37 (36S, 37S or 36R, 37R). The absolute configuration of this fragment was further determined by DP4 calculations (Marcarino et al., 2021). NMR shielding constants were computed using the GIAO method at the mPW1PW91/6-311 + G** level in the gas phase by the GAUSSIAN09 program (Zanardi et al., 2018). In the application of the DP4+ analysis method, we selected the only partial data of the 36,37,38-trihydroxybutyl moiety to carry out DP4 calculations of the two possible configurations (36S, 37S and 36R, 37R). Based on the calculation results, compound 1a (36S, 37S) showed satisfying linear regression analysis (R² = 0.9985) of the experimental data and calculated \(^13\)C chemical shifts (Figure 4), indicated better fit by comparison of experimental and calculated NMR data (Table 3), and then was designated as the most promising candidate. Therefore, the structure of compound 1 was assigned as shown in Figure 2.

Compound 2, pale-yellow powder, had the molecular formula of C_{38}H_{54}N_{2}O_{9} as based on its HRESIMS data [m/z 705.3724 (M + Na)]\(^+\), calculated for C_{38}H_{54}N_{2}O_{9}Na 705.3727, suggesting 13 degrees of unsaturation. The \(^1\)H NMR and \(^13\)C-APT NMR
spectra data (Table 1) showed resemblance with those of 1, except that compound 2 possessed an additional double bond at δC 122.8 and 150.2. In HMBC, the appearance of α–β unsaturated ketone at C-22–C-23 was further verified by the cross-peaks of H-23 (δH 7.34, 1H, d, J = 15.8 Hz), H-24 (δH 7.41, 1H, d, J = 15.8 Hz) to C-22 (δC 204.7) and correlations of the signals at H3-26 (δH 8.64, s) to C-3 (δC 157.3) and C-4 (δC 42.9) proved that the fragment of the 36,37,38-trihydroxybutyl unit was linked to C-32. Similar NOESY correlations and identical CD spectra of compounds 3 and 1 suggested their undifferentiated absolute configuration. Thus, the structure of 3 was elucidated as shown.

Compound 4 was obtained as a pale-yellow powder. The molecular formula of 4 was established to be C30H46O7 as deduced on the basis of (M + Na)+ ion peak at m/z 541.3126 (calculated C30H46O7Na 541.3141) in HRESIMS, which indicated eight degrees of unsaturation. The 1H NMR data (Table 1) of 4 displayed high similarity to those of 3, except for an additional double bond at the side chain, which indicated that compound 4 was a dehydrogenated product of compound 3, as proven by the HMBC experiment. In the HMBC spectrum, the cross peaks from H-23 (δH 7.35, 1H, d, J = 15.8 Hz) and H-24 (δH 7.41, 1H, d, J = 15.8 Hz) to C-22 (δC 204.8) and from H3-26 (δH 8.64, s) to C-3 (δC 157.3) and C-4 (δC 42.9) supported this deduction. The identical CD spectra and similar optical rotation of compounds 3–4 indicated that they owed the same absolute configuration. Therefore, the structure of compound 4 was determined as depicted and given the trivial name siragrosvenin D.

Compound 5 was isolated as a white amorphous powder, and it has a molecular formula of C38H54N2O9 as deduced on the basis of (M + Na)+ ion peak at m/z 707.3880 (calculated for C38H54N2O9Na 707.3883). Extensive analysis of its NMR data indicated that the structure of 5 was similar to that of 1, except for the downfield shifts of C-33 (Δδ + 0.8) and C-3 (Δδ + 1.6) and upfield shifts of C-32 (Δδ–0.9) and C-2 (Δδ–1.5), which indicated that the location of the trihydroxybutyl group may be different. By the HMBC experiment, the cross-peaks from H-35 (δH 3.79, 3.42) to C-32 (δC 143.3), C-33 (δC 154.6), and C-2 (δC 149.9) and from H-33 (δH 8.64, s) to C-3 (δC 157.3) and C-4 (δC 42.9) proved that the fragment of the 36,37,38-trihydroxybutyl unit was linked to C-32. Similar NOESY correlations and identical CD spectra of compounds 3 and 1 suggested their undifferentiated absolute configuration. Thus, the structure of 3 was elucidated as shown.

Compound 6 was obtained as a white amorphous powder, and it has a molecular formula of C30H46O7 as deduced on the basis of (M + Na)+ ion peak at m/z 541.3126 (calculated C30H46O7Na 541.3141) in HRESIMS, which indicated eight degrees of unsaturation. The 1H NMR data (Table 1) of 4 displayed high similarity to those of 3, except for an additional double bond at the side chain, which indicated that compound 4 was a dehydrogenated product of compound 3, as proven by the HMBC experiment. In the HMBC spectrum, the cross peaks from H-23 (δH 7.35, 1H, d, J = 15.8 Hz) and H-24 (δH 7.41, 1H, d, J = 15.8 Hz) to C-22 (δC 204.8) and from H3-26 (δH 8.64, s) to C-3 (δC 157.3) and C-4 (δC 42.9) supported this deduction. The identical CD spectra and similar optical rotation of compounds 3–4 indicated that they owed the same absolute configuration. Therefore, the structure of compound 4 was determined as depicted and given the trivial name siragrosvenin D.
213.2), one pair of double bonds ($\delta_{C}$ 142.9 and 119.0), three oxygenated methines ($\delta_{C}$ 81.8, 71.3, and 70.8), two oxygenated quaternary carbons ($\delta_{C}$ 72.6 and 110.0), two oxygenated methylenes ($\delta_{C}$ 71.1 and 64.8), and six methyls ($\delta_{C}$ 20.2, 21.0, 21.7, 22.6, 25.8, and 29.0). Based on these data, compound 5 was classified as a cucurbitane triterpenoid (Clericuzio et al., 2004).

Comparing the results of $^1$H and $^{13}$C NMR spectrum data of compound 5 with those of jinfushanencin F suggested their identical A/B/C/D rings (Li et al., 2016), except for the specific quaternary carbon signal at $\delta_{C}$ 110.0 in compound 5. Considering the unsaturation of 5, we inferred that there was an extra ring in the side chain. In the 2D NMR spectra, the fragment of C-24–C-25 (-C-27)-C-26 was established in the $^1$H-$^1$H COZY experiment, while the HMBC correlations from H-27 ($\delta_{H}$ 4.22, 4.09) and H-24 ($\delta_{H}$ 2.33, 1.71) to $\delta_{C}$ 110.0 suggested the presence of a spiro ring as formed by aldol condensation at C-23 (Figure 5). Thus, the planar structure of 5 was elucidated as an uncommon triterpenoid structure, which showed the spiro ring in the side chain.

The relative configuration was elucidated based on the NOESY correlations. Intense correlation of H-2/H-10 and H-3/Me-19 indicated that 2-OH was in $\beta$-orientation and 3-OH was in $\alpha$-orientation. In addition, the cross peaks of H-17/Me-30, H-10/Me-30, H-8/Me-18, and H-8/Me-19 suggested the $\beta$-orientation for H-10, Me-30, and H-17 and $\alpha$-orientation for H-8, Me-18, and Me-19. Fortunately, a suitable crystal of 5 was obtained and the single-crystal X-ray diffraction analysis was performed using Cu–Kα radiation (Figure 6), which established the absolute configuration of 5 to be $25S, 3S, 8S, 9R, 10R, 13R, 14S, 16R, 17R, 20R$, and $25S$. Consequently, the structure of 5 was named as siragrosvenin E.

Compound 6 was obtained as a white amorphous powder and given the molecular formula of C$_{30}$H$_{48}$O$_{7}$ based on HRESIMS. The $^1$H NMR spectrum of 6 also showed signals of a typical cucurbitacin triterpenoid with seven methyls at $\delta_{H}$ 1.27, 1.29, 1.32, 1.39, 1.49 (overlap), and 1.79 and two olefin protons at $\delta_{H}$ 5.15 (1H, m, H-23) and 6.06 (1H, m H-24). In addition, there were four oxygenated methines at $\delta_{H}$ 4.12 (1H, overlap, H-2), 3.45 (1H, d, $J$ = 9 Hz, H-3), 4.92 (1H, m, H-16), and 5.15 (1H, m, H-23) and one methylene at $\delta_{H}$ 4.30 (2H, s, H-26). Its $^{13}$C NMR spectra exhibited thirty carbon signals ascribed to seven methyls, five methylenes, four alkene carbon atoms, six oxygenated carbons, and five quaternary carbons (a carbonyl and an oxygenated carbon). The spectroscopic data displayed a resemblance to those of the known compound jinfushanencin F (Li et al., 2016), except for the more 18 amu than jinfushanencin F, which indicated that compound 6 could be a hydrolyzate of jinfushanencin F in the C-16–O-23 moiety. The key HMBC correlations from H-23 ($\delta_{H}$ 5.15) to C-20 ($\delta_{C}$ 71.8), C-22 ($\delta_{C}$ 50.0), C-24 ($\delta_{C}$ 126.2), and C-25 ($\delta_{C}$ 138.5) suggested that one hydroxy group was connected to C-23, and HMBC correlations...
from H-16 (δ_H 4.92) to C-13 (δ_C 49.1), C-14 (δ_C 48.9), C-17 (δ_C 56.3), and C-20 (δ_C 71.8) showed that another hydroxy group was attached to C-16 (Figure 5). The relative configurations of 6 were similar to those of compound 5 according to their similar NOESY correlations and NMR data. The ECD spectrum of 6 also showed same cotton effects to those of 5, suggesting identical absolute configuration. However, with rotational freedom in the side chain, it was not possible to definitively assign the C-23 configuration for either epimer. Thus, the structure of 6 was elucidated as depicted, named siragrosvenin F.

In addition, eight known compounds were isolated from the roots of S. grosvenorii. Their structures were identified as 23,24-dihydrocucurbitacin F (7) (Guerrero-Analco et al., 2007), cucurbitacin IIa (8) (Zeng et al., 2021), cucurbitacin Q1 (9) (Add El-Fattah, 1994), jinfushanencin F (10) (Li et al., 2016), siraitic acid A (11) (Si et al., 1999), siraitic acid B (12) (Si et al., 1999), cucurbitacin E (13) (Attard et al., 2005), and 23,24-dihydrocucurbitacin E (14) (Tang et al., 2015).

Proposed Biosynthetic Pathways for the Formation of Compounds 1–4

As far as we know, none of the identified cucurbitane-type pyrazine triterpenoid alkaloids has been reported as natural products so far in the literature. Consequently, we tried to deduce the potential biosynthetic pathway of compound 1 (Figure 7). First, the free amino acids in plants could react with the carbonyl group of hexose sugar. After the Strecker reaction and dehydration reaction, we obtained the amide-type conjugation (A) (Fujii and Kobatake, 1972; Wakamatsu et al., 2019). Simultaneously, 2, 3-oxysqualene was protonated, cyclized, rearranged, and deprotonated under the catalysis of various 2,3-oxidosqualene cyclases (OSCs) to obtain triterpenoid precursors, such as cucurbitenol. Then, cucurbitacin Iia was obtained under the catalysis of various cytochrome oxygenases P450 (CPY450), and the oxidation of its C-2 hydroxyl group will produce the derivative iso-23,24-dihydrocucurbitacin B (B), which could trigger the next chemical reaction. Subsequently, the precursor (B) was further aminated to form the structure C. The formation process of the pyrazines can be explained by the occurrence of Schiff’s base reaction by the degradation of the carbonyl...
group and amide group (Ganesan, 1996), which was easier to take place in the plants to yield compound 1. Finally, structures 2–4 could be generated by a similar mechanism as that described for structure 1.

**Cytotoxicity Evaluation of all Isolates**

Further studies were also performed using the MTT viability assay to evaluate the cytotoxicity of compounds 1–14 against MGC-803 (human gastric cancer cells), MCF-7 (human breast cancer cells), and CNE-1 (human nasopharyngeal carcinoma cells), and taxol (diterpene alkaloid) was used as a positive control. As shown in Table 4, compounds 4, 8, 9, 13, and 14 exhibited obvious *in vitro* cytotoxicity, with IC₅₀ values ranging from 1.44 to 9.99 μM. Cucurbitacin E, with the lowest IC₅₀ values among those compounds, has been investigated extensively for its cytotoxic activities toward several cancer cell lines through various underlying molecular mechanisms (Sun et al., 2010; Zhang et al., 2012; Attard and Martinoli, 2015). Through the analysis of the structure–activity relationship, α–β unsaturated ketone as functional groups can significantly enhance the cytotoxicity of cucurbitane-type compounds (Lin et al., 2015). In addition, compound 4 also showed the potential of cytotoxicity with an IC₅₀ of 8.04 μM.

**Cytotoxicity Against MCF-7 Cells of Siragrosvenin D (4)**

Cucurbitacin E, as the most anticancer potential natural products of the isolated compounds, has been investigated extensively for its cytotoxic activities toward several cancer cell lines through the various underlying molecular mechanisms (Huang et al., 2012; Attard and Martinoli, 2015). Therefore, to investigate the cytotoxicity of the siragrosvenin D, the cells were exposed to different concentrations of this compound. In cell cycle analysis,
siragrosvenin D significantly arrested the growth of cells at the G2/M phase, increasing from 12.70% of cells treated with the negative control to 13.44, 36.25, and 41.56% of cells treated with 5, 10, and 20 μM of siragrosvenin D, respectively (Figure 8). These results implied that siragrosvenin D inhibited proliferation of MCF-7 cells via the induction of G2/M phase arrest.

In order to study the mechanism of the promoting effect of siragrosvenin D on cell apoptosis, first, we observed the morphology of the cells after being treated with siragrosvenin D (0, 5, 10, and 20 μM). Comparing to negative control, with the increase of the concentration of siragrosvenin D, the number of cells started to decrease, accompanied with contraction and exfoliation (Figure 9). The results of AO/EB staining also demonstrated that siragrosvenin D significantly increased the percentage of apoptotic cells in the treated cells, which showed red fluorescence. Moreover, cell apoptosis was analyzed by flow cytometry. As shown in Figure 10, siragrosvenin D induced apoptosis of MCF-7 cells evidently. The percentage of apoptotic and necrotic cells in the treated cells was increased in a dose-dependent manner compared with that in the control group.

To assess the effect of siragrosvenin D on the proliferation of MCF-7 cells, we treated the cells with different concentrations of siragrosvenin D (0, 5, 10, and 20 μM) and evaluated its cell viability by the MTT assay for 24 and 48 h. It was observed that the growth of MCF-7 cells was suppressed in a dose- and time-dependent manner (Figure 11A). In addition, colony formation assay showed that after 14 days of incubation with siragrosvenin D, the number of colonies in the treated groups was significantly less than that in the control group (Figure 11B).

CONCLUSION

In summary, four novel cucurbitane-type triterpenoid pyrazine alkaloids, siragrosvenins A–D (1–4), along with two new cucurbitacins, siragrosvenins E–F (5–6), were isolated from the roots of S. grosvenorii. Among them, compounds 1–4 contained a novel cucurbitane-type triterpenoid skeleton with an additional pyrazine unit via a carbon–nitrogen linkage in the structure. Compound 5 showed an unexpected triterpenoid structure with a 6/6/6/5/6/5-fused polycyclic ring system, through aldol condensation. Although the pyrazine moiety is ever reported in plants (Li et al., 2006), siragrosvenins A–D (1–4) are the first examples of cucurbitane-type triterpenoid pyrazine alkaloids isolated from the herbs and may provide new chemotypes for the development of novel promising anticancer agents. Siragrosvenin B
and D showed more significant cytotoxicity against the tested cell lines, which further confirmed that the presence of the α,β-unsaturated ketone moiety could improve the antitumor activity. A widely accepted mechanism for these compounds was the occurrence of the Michael addition between the α,β-unsaturated ketone fraction and the soft nucleophiles, such as mercapten and protein sulfhydryl groups, resulting in the inactivation of the SH enzyme or SH coenzyme (Wijeratne et al., 2012).

Furthermore, we also conducted a preliminary investigation on siragrosvenin D, and the results implied that siragrosvenin D inhibited proliferation of MCF-7 cells and reduced their viability via the induction of G2/M phase arrest and significantly induced apoptosis in MCF-7 cells.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are publicly available. These data can be found here: https://www.ccdc.cam.ac.uk/structures/, 2151134.

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**AUTHOR CONTRIBUTIONS**

HJW: methodology and writing—original draft. GM: writing—review and editing. HQW: formal analysis. LI: formal analysis. AD: data curation. HL: validation. XH: software. JS: supervision and writing—review and editing. JH: supervision and writing—review and editing.

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**SUPPLEMENTARY MATERIAL**

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