A new alkaloid with cytotoxic activity from *Fritillaria thunbergii* Miq

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**ABSTRACT**

A new alkaloid named zhebeisine (1), together with four known compounds, eduardine (2), zhebeirine (3), isoverticine (4), and verticine (5), was isolated from the bulbs of *Fritillaria thunbergii* Miq. The structure of the new compound was elucidated on the basis of extensive spectroscopic methods and the *in vitro* biological activities of it were evaluated as well. Compound 1 features a veratramine skeleton with a rare 6/6/5/6/6/6 fused-ring system, representing the first reported veratramine-type alkaloid with a new oxazinane ring (ring-F) in *Fritillaria* genus. The cytotoxic activities study revealed that compound 1 inhibited the cell proliferation of HT29 and DLD1 (IC50 values of 25.1 and 48.8 μM, respectively) and also induced apoptosis of the above-mentioned two cancer cell lines.
1. Introduction

*Fritillaria thunbergii* Miq., an endemic plant in Zhejiang Province, China, is a rich source of steroidal alkaloids and has attracted plenty of research on their elaborate structures as well as their potential bioactivities. Previous phytochemistry investigations on *Fritillaria* plants focused on generous alkaloids (Yang and Duan 2012; Shou et al. 2010; Hu et al. 2018), many of which exhibited anticancer (Zhang et al. 2008), anti-inflammatory (Wang et al. 2016), anti-oxidant (Liu et al. 2017) and tracheal relaxant effects (Li et al. 2016). In the course of our research on active ingredients from *Fritillaria thunbergii* Miq., a new alkaloid named zhebeisine together with four known alkaloids was disclosed. Compound 1 (Figure 1) features a veratramine skeleton with a rare fused-ring system, representing the first reported veratramine-type alkaloid with a new oxazinane ring (ring-F) in *Fritillaria* genus. The structure of this new compound was elucidated by spectroscopic methods and its *in vitro* cytotoxic activities were evaluated. The structures of known alkaloids were determined as eduardine (2), zhebeirine (3), isoverticine (4), and verticine (5). Herein, we reported the isolation, structural elucidation, and biological activities of these compounds.

2. Results and discussion

Compound 1 was obtained as white amorphous powder and gave a positive reaction to Dragendorff’s reagent. It was shown to possess the molecular formula C_{28}H_{43}NO_{3} by HR-ESI-MS (m/z 442.3320 [M + H]{\textsuperscript{+}}, calcd. for C_{28}H_{44}NO_{3}, 442.3321), corresponding to eight degrees of unsaturation. IR spectrum had prominent absorption bands at 3400 and 1708 cm\(^{-1}\), characteristics for a hydroxyl group and a carbonyl group. The \(^1\)H-NMR spectrum of compound 1 displayed characteristic signal of two angular methylys \([\delta_{{\text{H}}} 1.61, s, Me-18; \delta_{{\text{H}}} 0.69, s, Me-19]\), a secondary methyl \([\delta_{{\text{H}}} 0.86, d, J = 6.3\text{ Hz, Me-27}]\) and an O-bearing CH group proton \([\delta_{{\text{H}}} 3.57, m, H-3]\). The \(^{13}\)C-NMR and DEPT spectra exhibited a total number of 28 carbon signals including a pair of olefinic bonds \([\delta_{{\text{C}}} 125.7, C-12; 138.3, C-13]\), a carbonyl carbon \([\delta_{{\text{C}}} 210.7, C-6]\), one quaternary carbon signal \([\delta_{{\text{C}}} 38.6, C-10]\), nine tertiary carbon signals, twelve secondary carbon signals, and three primary carbon signals. Analysis above indicated that compound 1 carried a C-nor-D-homo steroid skeleton, but one additional CH\(_2\) group \([\delta_{{\text{C}}} 87.4, C-28]\), together
with an unusual CH$_2$ group [$\delta_c$ 70.2, C-21] was observed as well. Further detailed inspection of NMR spectra data revealed that the chemical structure of compound 1 was similar to that of 18, N-(epoxymethano)-veratrosine (Li et al. 2019), possessing a similar 6/6/5/6/6/6 ring-fused system with 28 carbons. In the HMBC spectrum of compound 1 (see Figure S1 in Supporting information), correlations of H-28/C-26, H-28/C-22, and the correlations of H-21/C-17, H-21/C-20, H-21/C-22, H-21/C-28 proved the existence of an unusual oxazinane ring (ring F) in compound 1. Additionally, the hydroxyl group is traditionally located at C-3, which was confirmed by the HMBC correlations of H-5/C-3, H-5/C-10 and H-5/Me-19. Furthermore, the position of C=O was confirmed at C-6 based on the HMBC correlations of H-5/C-6 and C=O was located at C-12/C-13 based on the HMBC correlations of H-9/C-12, H-11/C-10, H-11/C-13, H-14/C-12 and H-14/C-13.

In the NOESY spectrum of 1 (see Figure S2 in Supporting information), correlations of H-3/H-5$_a$, H-19$_b$/H-8, H-5$_a$/H-9 and H-9/H-14 indicated that the positions of H-3, H-8, H-9, H-14 are $\alpha$-, $\beta$-, $\alpha$-, and $\alpha$-orientated, respectively. Furthermore, the correlations of H-14/H-16$_a$ and H-16$_b$/H-17 indicated the $\beta$-orientation of H-17. The correlations of H-17/H-26$_a$, H-26$_b$/Me-27 suggested the $\beta$-orientation of Me-27, and the correlations of H-17/H-23$_a$, H-23$_b$/H-22 suggested the $\beta$-orientation of H-22. Moreover, Me-18 correlating with H-20 suggested the $\beta$-orientation of H-20. Therefore, from the above analysis and biogenic synthesis pathway of this kind of compound, compound 1 was determined as (17$S$, 20$S$, 22$R$)-21, 28-oxazinane-veratraman-3$\beta$-hydroxyl-6-one-12-en and named as zhebeisine.

A possible biogenetic pathway for zhebeisine was described in Scheme 1. Peimisine, a natural product usually isolated from *Fritillaria* plants (Zhang et al. 2011; Won et al. 2018), opened its furan ring to form 6. 6 was converted into 7 by N-methylation, and 7 was oxidized to form 8. 8 was subjected to etherification to form 1.

The four known compounds were identified as eduardine (2) (Nuriddinov and Yunusov 1969), zhebeirine (3) (Zhang et al. 1991), isoverticine (4), and verticine (5) (Ito et al. 1963), respectively, by comparing the spectra data ($^1$H- and $^{13}$C-NMR) with those reported in the literature.

Scheme 1. Possible biogenetic pathway for zhebeisine (1).
The antiproliferative activity study of compound 1 was conducted on fourteen cancer cell lines. The results indicated that (see Table S1 in Supporting information) human colorectal cancer cell lines HT29 and DLD1 were inhibited most strongly (IC\textsubscript{50} values of 25.1 and 48.8 \(\mu\)M, respectively). 5-Fu was used as the positive control with IC\textsubscript{50} of 0.13 ± 6.1, 1.78 ± 1.9 to HT29 and DLD1, respectively. Furthermore, clone formation assay indicated that compound 1 had potential of inhibiting clone formation of HT29 cells (see Figure S3 in Supporting information). And apoptosis assay demonstrated that compound 1 induced the apoptosis of HT29 cells in a concentration-dependent manner (see Figure S4 in Supporting information).

3. Experimental part

3.1. General experiment

Column chromatography was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Factory, China). RP-18 was performed on reverse silica gel (20–45 \(\mu\)m; Mitsubishi Chemical Corporation, Japan). Sephadex LH-20 was from Pharmacia Fine Chemical Co., Ltd., Sweden. UV spectra were recorded using a UV3600 spectrophotometer (Shanghai Pharmaceutical Machinery Co. Ltd., China). IR spectra were recorded on a Nicolet 200 SXV spectrometer. Melting point was obtained from a tester (Jinan Halon Instruments, China). HR-ESI-MS data were performed on a Waters Q-TOF Premier mass spectrometer (Waters, USA). NMR spectra were obtained by an AC-E200 400 MHz digital NMR spectrometer (Bruker Corporation, Karlsruhe, German), using TMS as the internal standard.

3.2. Plant material

The bulbs of *Fritillaria thunbergii* Miq. were purchased in Chengdu Lotus Pond Market (Chengdu, China) and were identified by Associate professor Jianzhong Wang. The specimen (No. FT20190918) was deposited at the Department of Medicinal Natural Products, West China School of Pharmacy, Sichuan University.

3.3. Extraction and isolation

The bulbs (20 kg) of *Fritillaria thunbergii* Miq. were extracted completely with MeOH to give the extracts (1.3 kg) after concentration. The extracts were acidified with 0.1 mol/L hydrochloric acid solution to give the solid extract and acid solution. The later part was basified with ammonia aqueous to pH 9–10 and then was extracted with ethyl acetate to give the alkaloids fraction (201.0 g) after removing the solvent. The alkaloids fraction was subjected to normal CC eluting with PE-EA (20:1-1:1, v/v) to give fractions Fr. I-Fr. XIII. Fr. I (3.8 g) was subjected to silica gel CC eluting with DCM-EA (5:1-1:1, v/v) to produce five fractions (Fr. I-1-Fr. I-5), and then Fr. I-1 together with Fr. I-2 (1.1 g) was separated by silica gel CC repeatedly in gradient eluting system of DCM-acetone (12:1, v/v) to obtain compound 1 (90.3 mg). Fr. II together with Fr. III (8.2 g) was eluted with DCM-acetone to give three fractions, and the third fraction was subjected to silica gel CC eluting with DCM-acetone (5:1, v/v) to give compound 2 (293.5 mg) and
compound 3 (240.3 mg). Fr. IV (5.6 g) was eluted with DCM–MeOH (100:0.1–100:2, v/v) on silica gel CC to give five fractions Fr. IV-1 to Fr. IV-5, and Fr. IV-3 was eluted with PE-acetone (3:1, v/v) to obtain compound 5 (221.6 mg). Fr. VI (1.3 g) was subjected to silica gel CC eluting with PE-acetone (15:1-1:1, v/v) and then separated by Sephadex LH-20 to obtain compound 4 (20 mg).

Zhebeisine (1): White amorphous powder, [α]D25 = +1.53 (c = 0.18, CHCl3), UV (MeOH) λmax (log ε) 208 (3.96) nm; IR (KBr) νmax: 3400, 1708 cm⁻¹; 1H-NMR (400 MHz, CDCl3): δ 1.62 (1H, m, H-1a), 1.33 (1H, m, H-1b), 1.92 (1H, m, H-2a), 1.76 (1H, m, H-2b), 3.54 (1H, m, H-3), 1.81 (1H, m, H-4a), 1.42 (1H, m, H-4b), 2.19 (1H, dd, J = 12.5, 2.9 Hz, H-5), 2.12 (1H, dd, J = 13.1, 4.6 Hz, H-7a), 2.52 (1H, dd, J = 13.1, 4.6 Hz, H-7b), 1.37 (1H, dd, J = 12.0, 3.8 Hz, H-8), 1.73 (1H, m, H-9), 2.34 (1H, dd, J = 16.7, 8.4 Hz, H-11a), 1.90 (1H, dd, J = 16.7, 8.4 Hz, H-11b), 1.78 (1H, m, H-14), 0.70 (1H, m, H-15a), 1.80 (1H, m, H-15b), 1.77 (1H, m, H-16a), 1.42 (1H, m, H-16b), 2.28 (1H, br.s, H-17), 1.62 (3H, s, H-18), 0.68 (3H, s, H-19), 2.01 (1H, m, H-20), 3.26 (1H, t, J = 11.2 Hz, H-21a), 3.63 (1H, dd, J = 11.2, 3.5 Hz, H-21b), 2.03 (1H, m, H-22), 1.91 (1H, m, H-23a), 1.25 (1H, m, H-23b), 0.94 (1H, m, H-24a), 1.79 (1H, m, H-24b), 1.92 (1H, m, H-25), 1.59 (1H, d, J = 10.9 Hz, H-26a), 2.66 (1H, d, J = 10.9 Hz, H-26b), 0.86 (3H, d, J = 6.3 Hz, H-27), 3.57 (1H, d, J = 7.8 Hz, H-28a), 4.35 (1H, d, J = 7.8 Hz, H-28b); 13C-NMR (100 MHz, CDCl3): δ 37.2 (C-1), 30.6 (C-2), 71.0 (C-3), 30.2 (C-4), 56.9 (C-5), 210.7 (C-6), 46.0 (C-7), 47.3 (C-8), 54.4 (C-9), 38.6 (C-10), 28.5 (C-11), 125.7 (C-12), 138.3 (C-13), 47.8 (C-14), 26.0 (C-15), 23.0 (C-16), 35.4 (C-17), 17.4 (C-18), 12.6 (C-19), 42.4 (C-20), 70.2 (C-21), 63.3 (C-22), 28.1 (C-23), 33.1 (C-24), 30.5 (C-25), 57.1 (C-26), 19.8 (C-27), 87.4 (C-28); HR-ESI-MS m/z 442.3320 [M + H]+, calcld. for C28H44NO3, 442.3321).

3.4. Antiproliferative activity

Seven colorectal cancer lines (HT29, DLD1, HCT116, HCT115, OV5, SW480, SW620), two liver cancer lines (HepG2, JHH7), one breast cancer line (MCF-7), one lung cancer line (A549), and three ovarian cancer lines (Skov3, A2780, ES2) selected from the human cancer cell lines were used in antiproliferative activity study. All cells were cultured in 5% CO₂ at 37°C in RPMI-1640 or DMEM which was supplemented with 10% fetal bovine serum (FBS). The in vitro cytotoxicity of these cells was measured by the MTT method (Mosmann 1983). Briefly, 100 µL of the cell suspension was seeded into each well of 96-well cell culture plates at a density of 3 × 10³ cells/well, and the plates were allowed to incubate for 12 h prior to adding test samples. Then, 100 µL test samples of different concentrations were added to the well for another 48 h. After treatment, 20 µL of MTT solution in PBS (Phosphate buffer) was added to each well to incubate at 37°C for 4 h. Then, 100 µL of DMSO was added to each well. Finally, the absorbance was determined at 570 nm using a spectrophotometer. The half-maximal inhibitory concentration (IC₅₀) values were calculated using GraphPad Prism software. And all the experiments were repeated in triplicate.

3.5. Plate clone formation assay

For the assay (Plumb 2004), 500 – 1000 cells were added into each well of 12-well plates and the wells were treated with different concentrations of compound 1 (2.5, 5,
10 μM) for 14 days. After washed with PBS, 1 mL paraformaldehyde fixing solution (4%) was added into each well for 15 min. Then, washed with PBS for three times, the cells were stained with crystal violet dye solution for 30 min. The cells were washed with PBS for several times and dried naturally, and the number of cell clones (≥50 cells) was counted.

3.6. Cellular apoptosis assay

The cellular apoptosis was determined by previously reported method (Rieger et al. 2011). Briefly, the HT29 cells were seeded in a 6-well plate at a density of 3 × 10^5 cells/well and incubated for 24 h at 37°C. Then, cells were treated with different concentrations of compound 1 (20, 40, 60 μM) and incubated for 48 h. To assess the cellular apoptosis, the treated cells were collected, washed with PBS for three times and stained with the Annexin V-FITC and PI at room temperature for 15 min in the dark. Finally, the cells were analyzed by flow cytometry. The minimum number of cells analyzed for each sample was 10000.

3.7. Statistical analysis

The experimental results were expressed as mean ± SD. Data analysis was performed using the one-way ANOVA, and a p-value <0.05 was considered to be significant difference.

4. Conclusions

In this work, one new alkaloid and four known alkaloids were isolated from *Fritillaria thunbergii* Miq. To the best of our knowledge, compound 1 represents the first alkaloid that possesses a veratramine skeleton with a unique 6/6/5/6/6/6 fused-ring system in *Fritillaria* genus. Besides, it showed potential antitumor activities. Thus, our findings would be useful for further investigations into the secondary metabolites of *Fritillaria thunbergii* Miq.

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Disclosure statement

The authors declare no conflicts of interest.

References

Hu Z, Zong JF, Yili A, Yu MH, Aisa HA, Hou AJ. 2018. Isosteroidal alkaloids from the bulbs of *Fritillaria tortilofila*. Fitoterapia. 131:112–118.

Ito S, Kato M, Shibata K, Nozoe T. 1963. On the alkaloid of *Fritillaria verticillata* WILD. var. *Thunbergii* BAKER. II. The structure of verticine. Chem Pharm Bull. 11(10):1337–1340.
Li Q, Long CB, Zhu PF, Liu YP, Luo XD. 2019. Seven new veratramine-type alkaloids with potent analgesic effect from *Veratrum taliense*. J Ethnopharmacol. 244:112137.

Li Y, Yili A, Li J, Muhamat A, Aisa HA. 2016. New isosteroidal alkaloids with tracheal relaxant effect from the bulbs of *Fritillaria pallidiflora* Schrenk. Bioorg Med Chem Lett. 26(8):1983–1987.

Liu YM, Feng YD, Lu X, Nie JB, Li W, Wang LN, Tian LJ, Liu QH. 2017. Isosteroidal alkaloids as potent dual-binding site inhibitors of both acetylcholinesterase and butyrylcholinesterase from the bulbs of *Fritillaria walujewii*. Eur J Med Chem. 137:280–291.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65(1–2):55–63.

Nuriddinov RN, Yunusov SY. 1969. Structure and configuration of edpetilidine and eduardine. Chem Nat Compd. 5(4):284–285.

Plumb JA. 2004. Cell sensitivity assays: clonogenic assay. Methods Mol Med. 88:159–164.

Rieger AM, Nelson KL, Konowalchuk JD, Barreda DR. 2011. Modified Annexin V/propidium iodide apoptosis assay for accurate assessment of cell death. J Visualized Exp. 50:2597

Shou QY, Tan Q, Shen ZW. 2010. Two 22S-solanidine-type steroidal alkaloids from *Fritillaria anhuiensis*. Fitoterapia. 81(2):81–84.

Wang D, Yang J, Du Q, Li H, Wang S. 2016. The total alkaloid fraction of bulbs of *Fritillaria cirrhosa* displays anti-inflammatory activity and attenuates acute lung injury. J Ethnopharmacol. 193:150–158.

Won SS, Seung YL, Jong EP, Dong HK, Sun YK, Kang RL. 2018. Two new steroidal alkaloids from the bulbs of *Fritillaria thunbergii*. Heterocycles. 96(5):921–930.

Yang ZD, Duan DZ. 2012. A new alkaloid from *Fritillaria ussuriensis* Maxim. Fitoterapia. 83(1):137–141.

Zhang JX, Ma GE, Nao AL, Xu RS. 1991. Studies on chemical constituents of *Fritillaria thunbergii* Miq. Acta Pharm Sin. 26(3):231–233.

Zhang YH, Zhang QJ, Zheng ZF, Yu DQ. 2011. Steroidal alkaloids from the bulbs of *Fritillaria unibracteata*. J. Asian Nat. Prod. Res. 13(12):1098–1103.

Zhang YH, Yang XL, Zhang P, et al. 2008. Cytotoxic Alkaloids from the Bulbs of *Fritillaria hupehensis*. Chem Biodivers. 5(2):259–266.