Two new ent-atisanes from the root of *Euphorbia fischeriana* Steud.

Meng Wang¹, QiuHong Wang¹, Qing Wei, Jing Li, Chen Guo, Bingyou Yang and Haixue Kuang*

Key Laboratory of Chinese Materia Medica (Ministry of Education), Heilongjiang University of Chinese Medicine, No. 24, Heping Road, Xiangfang District, Harbin 150040, P.R. China

(Received 20 December 2014; final version received 18 April 2015)

Two new ent-atisanes *ent*-1β,3β,16β,17-tetrahydroxyatisane (1), *ent*-1β,3α,16β,17-tetrahydroxyatisane (2) together with 11 known diterpenes were isolated from the anti-tumour activity fraction of *Euphorbia fischeriana* Steud. The compounds were identified by detailed spectroscopic analysis, including extensive 2D-NMR experiments. X-ray analysis was applied to determine the structure of compound 2. All 13 compounds were screened for cytotoxicity *in vitro* against human tumour MCF-7, HepG-2 and SGC-7901 cell lines. Compounds 1 and 2 showed the inhibitory effects against MCF-7 with IC₅₀ levels of 23.21 and 15.42 μM; simultaneously, compounds 4, 6, 8 and 11 also had definite inhibitory effect against different cell lines.

**Keywords:** ent-atisane; *Euphorbia fischeriana* Steud; X-ray; stereoismers; anti-tumour activity

1. Introduction

*Euphorbia fischeriana* Steud. is a perennial herbaceous plant distributed mainly in northern China. The root of *E. fischeriana* Steud. had been used for healing cancer, edema and tuberculosis in traditional Chinese Medicine for more than 2000 years, which has been supported in recent studies (Qin & Xu 1998; Sun & Liu 2011; Wang et al. 2013). The genus *Euphorbia* has been widely spread in the Euphorbiaceae family and a growing supply of experimental evidence that *Euphorbia* has a broad range of biological activities and medicinal value (Shi et al. 1999; Zhou et al. 2003). Intense anti-tumour activity in HepG-2 and MCF-7 of this plant had been suggested in previous chemical studies (Ma et al. 1997; Wang et al. 2006). Therefore, we commenced a further chemical study on the root of *E. fischeriana* Steud. focusing on diterpenes and completed the isolation of two new atisanes *ent*-1β,3β,16β,17-tetrahydroxyatisane (1) and *ent*-1β,3α,16β,17-tetrahydroxyatisane (2), as well as 11 known diterpenes including three atisanes, *ent*-3β,13S-dihydroxy-atis-16-en-14-one (3), *ent*-16α,17-dihydroxyatisan-3-one (4)

*Corresponding author. Email: hxkuang@hotmail.com

© 2015 Taylor & Francis
and ent-atisane-3β,16α,17-triol (5), one kaurane, 3S,16S,17-trihydroxy-2-one-ent-kaurane (6), three tiglianes, phorbol-13-actate (7), prostratin (8) and langduin A (9), four jolkinolides, jolkinolide A (10), 17-hydroxyjolkinolide A (11), jolkinolide B (12) and 17-hydroxyjolkinolide B (13) (Figure 1) from ethyl acetate extract of the root of *E. fischeriana* Steud. The cytotoxicity of all the 13 compounds were tested by MTT against the tumour cell lines MCF-7, HepG-2 and SGC-7901, and the result showed that diterpenes possessed anti-tumour activity and compounds 1 and 2 had valid inhibitory effect with IC$_{50}$ levels of 23.21 and 15.42 μM in MCF-7.

2. Results and discussion

Compound 1 was obtained as colourless needles. It had the molecular formula C$_{20}$H$_{34}$O$_{4}$ by HR-ESI-MS at $m/z$ 361.2254 [M + Na]$^+$ (calcd for C$_{20}$H$_{34}$O$_{4}$, 361.2258). The IR (KBr) spectrum indicated that 1 possessed hydroxyl group ($3452$ cm$^{-1}$). The $^1$H NMR(C$_5$D$_5$N, 400 MHz) spectrum of compound 1 clearly showed three methyls at $δ1.32$ (3H,s), $δ1.20$ (3H,s) and $δ1.10$ (3H,s). The $^{13}$C NMR (C$_5$D$_5$N, 100 MHz) and DEPT spectroscopic data revealed the presence of 20 carbon signals, including three methyls, eight methylenes, five methines and four quaternary carbons. In the HMBC spectrum, the correlation of H-18 and H-19 with C-3 ($δ75.9$), C-5 ($δ53.7$); H-20 with C-5 ($δ53.7$) and C-9 ($δ2.8$); H-17 with C-12 ($δ33.1$) and C-15 ($δ53.6$) was observed (Figure 2). The NOESY correlations helped assign the relative configuration of compound 1. The correlations of compounds 1 and 2 are similar, except that 1-OH, 3-OH and 16-OH have some differences. It has been confirmed that the orientations of H-5 was supposed to be $β$ while H-20 was supposed to be $α$ with comparison of compound 2. Correlations of H-3/H-5, H-1, H-19; H-5/H-9, H-1, H-3 and H-9/H-17, H-5 confirmed H-1, H-3, H-5, H-9, CH$_3$-19 to be

![Figure 1](image-url)
β. Correlations of H-20/H-14, H-18; H-14/H-12 confirmed H-12, CH₃-20, CH₃-18, H-14 to be α (Figure S17). Therefore, 1 was elucidated as ent-1β,3β,16β,17-tetrahydroxyatisane.

Compound 2 is the stereoisomer of 1. Compound 2 was obtained as colourless needles. It had the molecular formula C₂₀H₃₄O₄ by HR-ESI-MS at m/z 361.2240 [M + Na]⁺ (calcd for C₂₀H₃₄O₄, 361.2248). The IR (KBr) spectrum indicated that 2 possessed a hydroxyl group (3442 cm⁻¹). The ¹H NMR (C₅D₅N, 400 MHz) spectrum of compound 2 showed three methyls at δ1.34 (3H,s), δ1.17 (3H,s) and δ0.95 (3H,s). The ¹³C NMR (C₅D₅N, 100 MHz) and DEPT spectroscopic data revealed the presence of 20 carbon signals, including three methyls, eight methylenes, five methines and four quaternary carbons. In the HMBC spectrum, the correlation of H-18 and H-19 with C-3 (δ76.7), C-5 (δ48.6), C-4 (δ38.0); H-20 with C-1 (δ76.1) and C-9 (δ52.8); H-17 with C-12 (δ33.2), C-15 (δ53.8) and C-16 (δ73.7) was observed. The NOESY correlations helped to assign the relative configuration of compound 2. Correlations of H-5/H-1, H-9, H-19 and correlations of H-18/H-3, H-20; H-14/H-20, H-12 and H-17/H-13 were observed. To further determine the configuration of 2, a single crystal of 2 was analysed by X-ray crystallography.

The stereochemistry of compound 2 was analysed by X-ray. Suitable crystals were mounted on a glass fibre on a single-crystal X-ray diffractometer (German Bruker SMART 1000 CCD) operating at 50 kV and 40 mA using Mo Ka radiation (0.71073 Å). Data collection and reduction were performed using the SMART and SAINT software. The structure was solved by direct methods, and the non-hydrogen atoms were subjected to anisotropic refinement by full-matrix least squares on F² using SHELXTL package. All hydrogen atoms were located in their calculated positions and treated using a riding model. Compound 2: empirical formula C₂₀H₃₄O₄, formula weight 338.47, orthorhombic, space group, P 2₁ 2₁ 2₁, Z = 4, a = 7.1632(3) Å, b = 11.8779(5) Å, c = 22.0984(10) Å²; μ (MoKα) = 0.081 mm⁻¹, Dc = 1.196 Mg/m³; S = 1.067, final R factors: R₁ = 0.0387 and wR₂ = 0.0856 for 3317 observed from 5817 independent and 2983 measured reflections [θmax = 25.01, I > 2σ(I) criterion and 223 parameters]; maximum and minimum residues are 0.248 and −0.187 e Å⁻³, respectively. The Flack parameter is 0.0(4). Crystallographic data for the structural analysis have been deposited with the Cambridge Crystallographic data centre, information on CCDC No. 975836 may be obtained free of charge from the +44(1223) 336 033 or Email: deposit@ccdc.cam.ac.uk or www: http://www.ccdc.cam.ac.uk.

According to the data of X-ray and NOESY correlations, simultaneously compared with the literature (Allick et al. 1990; Zhan et al. 2013), the orientations of H-5 and H-20 have been determined as β and α, respectively, in atisane skeleton. Therefore, in compound 2, the orientations of H-1, H-5, H-9 and CH₃-19 have been confirmed as β while H-3, CH₃-18 and

Figure 2. Crystal structures of compound 2.
CH₃-20 have been determined as α. Compound 2 was elucidated as ent-1β,3α,16β,17-tetrahydroxyatisane.

By comparison of the physical and spectral data with literature values, the 11 known compounds (3–13) were identified as ent-3β,13S-dihydroxy-atis-16-en-14-one (3) (Wang et al. 2004), ent-16α,17-dihydroxyatisan-3-one (4) (Shi et al. 2005) and ent-atisane-3β,16α,17-tri­oil (5) (Jia & Ding 1991), 35,165,17-trihydroxy-2-one-ent-kaurane (6) (Zhang et al. 2012), phorbol-13-actate (7) (Wang et al. 2010), prostratin (8) (Tang et al. 2012), langduin A (9) (Ma et al. 1997), jolkinolide A (10) (Pan et al. 2004), 17-hydroxyjolkinolide A (11) (Liu et al. 1988), jolkinolide B (12) (Wu et al. 2010), 17-hydroxyjolkinolide B (13) (Zhao et al. 1994).

3. Experimental
3.1. Plant material
The root of Euphorbia fishcheriana Steud. was collected in Heilongjiang Province of China in September 2011, and identified by Prof. Zhenyue Wang (Heilongjiang University of Chinese Medicine). The voucher specimen (20110047) was deposited at the Herbarium of Heilongjiang University of Chinese Medicine, Harbin, China.

3.2. Extraction and isolation
The air-dried, powdered roots of Euphorbia fishcheriana Steud. (3.0 kg) were extracted under reflux conditions with 95% ethanol (3 L × 3 × 2 h each). The ethanolic solution was concentrated in vacuo to 250 g, which was dissolved in H₂O solution (2000 mL) and then portioned between EtOAc and n-BuOH successively to provide EtOAc-soluble (125 g) and n-BuOH-soluble (120 g) portions. The EtOAc-soluble portion was subjected to silica gel column chromatography (open column 150 cm × 25 cm) eluting with dichloromethane containing increasing amounts of methanol to obtain six fractions. Fr.1 (8 g) was further purified on ODS column chromatography (MeOH–H₂O 80:20) to afford 4 (23 mg), 5 (12 mg) and 6 (13 mg). Fr.3 (7 g) was isolated by sephadex LH-20 column using MeOH as eluent to yield 7 (9 mg), 8 (11 mg) and 9 (8 mg). Fr.5 (22 g) was further purified on column chromatography (silica gel, CH₂Cl₂: MeOH 5:1) to afford 10 (13 mg), 11 (10 mg) and 12 (11 mg). Fr 6 (13 g) was re-fractionated by Waters preparative HPLC (Waters 2535 pump equipped with Waters 2487 detector; column: Yilite Hypersil-ODS II, i.d. 10 μm, 20 × 300 mm; elution rate: 3.0 mL/min; column temperature: 30°C; solvent: MeOH/H₂O 45:55) to give a total of 12 sub-fractions (8 mL each). Sub-fractions 1–3 have been merged to get 1 (18 mg), sub-fractions 5–8 have been merged to get 2 (22 mg) and sub-fractions 10–11 have been merged to get 3 (15 mg).

3.2.1. ent-1β,3β,16β,17-Tetrahydroxyatisane (1)
Compound 1: [α]D²⁰ + 10.5 (c = 0.24, MeOH). HR-ESI-MS: m/z 361.2254 [M + Na]⁺ (calcd for C₂₀H₃₄O₄, 361.2258). IR: 3452,3430(OH). ¹H-NMR (pyridine-d₅, 400 MHz): δ 3.93(1H, d, J = 10.9, H-17), 3.82(1H, d, J = 10.9, H-17), 3.67-3.71(1H, t, J = 7.2, H-1), 3.55(1H, t, J = 8.5, H-3), 2.94(1H, td, J = 11.2,3.0, H-11), 2.31(1H, s, H-2), 2.29(1H, s, H-2), 2.26-2.28(1H, m, H-11),2.23-2.24(1H, m, H-12), 1.91-1.97(1H, m, H-14), 1.86-1.88(1H, m, H-9), 1.80-1.84(1H, m, H-13), 1.58-1.61(1H, m, H-15), 1.57(1H, d, J = 3.0, H-13), 1.43-1.49(1H, m, H-6), 1.41(1H, s, H-7), 1.38(1H, s, H-15), 1.32(3H, s, H-20), 1.22-1.25(1H, m, H-7), 1.20(3H, s, H-19), 1.10(3H, s, H-18), 0.86(1H, td, J = 12.4,6.2, H-14), 0.75-0.78(1H, m, H-5); ¹³C NMR data (pyridine-d₅, 100 MHz): δ 79.6(C-1), 75.9(C-3), 73.7(C-16), 69.5(C-17), 53.7(C-5), 53.6(C-15), 52.8(C-9), 43.5(C-10), 40.8(C-7), 39.6(C-4), 39.1(C-2), 33.6(C-8), 33.1(C-12), 28.7(C-19), 28.4(C-14), 27.1(C-11), 24.3(C-13), 18.5(C-6), 16.4(C-18), 11.3(C-20).
3.2.2. ent-1β,3α,16β,17-Tetrahydroxyatisane (2)

Compound 2: [α]D20 -19.1 (c = 0.28, MeOH). HR-ESI-MS: m/z 361.2240 [M + Na]+ (calcd for C20H34O4, 361.2248). IR: 3505, 3442(OH). 1H NMR (pyridine-d5, 400 MHz): δ 4.48(1H, dd, J = 11.4, 4.4, H-1), 3.90(1H, d, J = 10.9, H-17), 3.80(1H, d, J = 10.9, H-17), 3.75(1H, t, J = 2.6, H-3), 2.97(1H, td, J = 12.7,3.1, H-11), 2.36(1H, d, J = 2.5, H-2), 2.32(1H, td, J = 6.8,3.2, H-11), 2.23-2.24(1H, m, H-12), 2.19(1H, t, J = 3.8, H-2), 2.10(1H, dd, J = 10.7,6.9, H-9), 1.96(1H, t, J = 12.8, H-14), 1.84(1H, td, J = 12.8,2.9, H-13), 1.74(1H, d, J = 10.3, H-5), 1.60(1H, td, J = 6.3,2.6, H-13), 1.56(1H, d, J = 3.1, H-6), 1.51-1.54(1H, m, H-15), 1.45-1.48(1H, m, H-6), 1.39-1.40(1H, m, H-15), 1.37(1H, s, H-7), 1.34(3H, s, H-20), 1.25(1H, td, J = 13.6,4.3, H-7), 1.17(3H, s, H-19), 0.95(3H, s, H-18), 0.84(1H, td, J = 12.6,6.2, H-14); 13C NMR data (pyridine-d5, 100 MHz): δ 76.7(C-3), 76.1(C-1), 73.7(C-16), 69.6(C-17), 73.9(C-15), 52.8(C-9), 48.6(C-5), 43.5(C-10), 40.9(C-7), 38.0(C-4), 37.5(C-2), 33.8(C-8), 33.2(C-12), 29.1(C-19), 28.4(C-14), 27.1(C-11), 24.3(C-13), 22.8(C-18), 18.8(C-6), 11.1(C-20).

3.3. Cytotoxicity

The cytotoxicity of all 13 compounds was tested by MTT method (Wang et al. 2009, 2011). The result showed that compounds 1 and 2 had an intensive effect with IC50 levels of 23.21 and 15.42 µM in human MCF-7 cell line; compounds 4, 8 and 13 with IC50 levels of 23.12, 21.92 and 21.49 µM, respectively, in human HepG-2 cell line; compounds 4 and 13 with IC50 levels of 21.28 and 23.79 µM in human MCF-7 cell line and compound 4 with IC50 levels of 22.20 µM in human SGC-7901 cell line.

4. Conclusion

In our study, 13 diterpenes compounds were isolated from E. fischeriana Steud. and identified. Compounds 1 and 2 are two new ent-atisane compounds and stereoisomers for each other whose configuration was further determined by X-ray. Compounds 3, 4 and 6 were isolated from E. fischeriana Steud. for the first time. According to the result of MTT, different degrees of inhibition on three different human cell lines MCF-7, HepG-2 and SGC-7901 were indicated by compounds 1, 2, 4, 8 and 13, respectively.

Supplementary material

Experimental details relating to this article are available online, alongside Tables S1 and S2 and Figures S1–S17.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Basic Research Program of China [973 Program grant number 2013CB531801] and the Foundation of Research and Innovation for Graduate Student of Heilongjiang Province [grant number YJSCX2012-354HLJ].

Note

1. These authors contributed equally to this study.
References

Allick RL, Richard CC, Peter SR, Paul DW. 1990. *ent*-Atisane diterpenes from *Euphorbia fidijiana*. Phytochemistry. 29:1925–1935.

Jia ZJ, Ding YL. 1991. New diterpenoids from *Euphorbia sieboldiana*. Planta Med. 51:569–571.

Liu GF, Fu YQ, Yang ZQ, Zhao HQ, Fan XM. 1988. *Euphorbia* anticancer constituents isolation and identification of diterpene lactone. Tradit Chin Med Bull. 5:191–192.

Ma QG, Liu WZ, Wu XY. 1997. Diterpenoids from *Euphorbia fischeriana*. Phytochemistry. 44:663–666.

Pan Q, Shi MF, Min ZD. 2004. *Euphorbia* Jolkinolide type of four-dimensional NMR studies of diterpenoid. J China Pharm Univ. 35:16–19.

Qin GW, Xu RS. 1998. Recent advances on bioactive natural products from Chinese medicinal plants. Med Res Rev. 18:375–382.

Shi HM, Williams Ian D, Sung HH, Zhu HX, Ip NY, Min XD. 2005. Cytotoxic diterpenoids from the roots of *Euphorbia ebracteolata*. Planta Med. 71:349–354.

Shi Y, He Z, Jia Z. 1999. Progress in the structures of diterpenoids and the bioactives from *Euphorbia* genus. Nat Prod Res Dev. 11:85–89.

Sun YX, Liu JC. 2011. Chemical constituents and biological activities of *Euphorbia fischeriana* Steud. Chem Biodivers. 8:1205–1214.

Tang Q, Su ZH, Han ZT. 2012. LC-MS method for detecting prostratin in plant extracts and identification of a high-yielding population of *Euphorbia fischeriana*. Phytochem Lett. 5:214–218.

Wang HB, Chu WJ, Wang Y, Ji P, Wang YB, Yu Q, Qin GW. 2010. Diterpenoids from the roots of *Euphorbia fischeriana*. J Asian Nat Prod Res. 12:1038–1043.

Wang JH, Zhang K, Niu HY, Shu LH, Yue DM, Li D, He P. 2013. Jolkinolide B from *Euphorbia fischeriana* Steud. induces in human leukemic cells apoptosis via JAK2/STAT3 pathways. Int J Clin Pharmacol Ther. 51:170–178.

Wang JH, Zhou YJ, Bai X, Ping H. 2011. Jolkinolide B from *Euphorbia fischeriana* Steud. induces apoptosis in human leukemic U937 cells through PI3K/Akt and XIAP pathways. Mol Cells. 32:451–457.

Wang H, Zhang XF, Ma YB, Cai XH, Wu DG, Luo XD. 2004. Diterpenoids from *Euphorbia wallichii*. Chin Tradit Herb Drugs. 35:611–614.

Wang Y, Ma X, Yan S, Shen S, Zhu H, Gu Y, Wang HB, Qin G, Yu Q. 2009. 17-Hydroxy-jolkinolide B inhibits signal transducers and activators of transcription signaling by covalently cross-linking Janus kinases and induces apoptosis of human cancer cells. Cancer Res. 69:7302–7310.

Wang YB, Huang R, Wang HB, Jin HZ, Lou LG, Qin GW. 2006. Diterpenoids from the roots of *Euphorbia fischeriana*. J Nat Prod. 69:967–970.

Wu QC, Tang YP, Ding AW, You FQ, Duan JA. 2010. Diterpenes and triterpenes from the roots of *Euphorbia fischeriana*. Chin J Nat Med. 8:101–103.

Zhang BY, Wang H, Luo XD, Du ZZ. 2012. Two novel diterpene dimers from the roots of *Euphorbia yinshanica*. Helv Chim Acta. 91:1672–1679.

Zhao KJ, Xu GJ, Jin RL, Xu LS, Cong XD. 1994. *Euphorbia* crude drug Jokinolide B HPLC analysis. J China Pharm Univ. 25:332–334.

Zhan R, Li XN, Du X, Wang WG, Dong K, Su J, Li Y, Pu JX, Sun HD. 2013. *ent*-Atisane and *ent*-kaurane diterpenoids from *Isodon rosthornii*. Fitoterapia. 88:76–81.

Zhou TX, Bao GH, Ma QG, Qin GW, Che CT, Lv Y, Wang C, Zheng QT. 2003. Langduin C, a novel dimeric diterpenoid from the roots of *Euphorbia fischeriana*. Tetrahedron Lett. 44:135–137.