Fimbriatols A–J, Highly Oxidized ent-Kaurane Diterpenoids from Traditional Chinese Plant Flickingeria fimbriata (B1.) Hawkes

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Fimbriatols A–J (1–10), ten new ent-kaurane diterpenoids possessing differently highly oxidized sites, were isolated from Flickingeria fimbriata (B1.) Hawkes. The structures of these new compounds were determined by HRESI-MS, NMR, CD spectra and X-ray diffraction analysis. Compound 1 displayed moderately inhibitory ratio (48.5%) compared with the positive compound NSC-87877 (81.6%) at the concentration of 0.022 μg/mL. Compounds 7–10 possess 3, 4-seco-ent-kaurane skeleton containing a disaccharide moiety with an unusual linkage at C-2′ to C-1″ instead of the common linkage at C-6′ to C-1″, and this is the first report in 600 more ent-kauranes found in nature, which might be originated from ent-kaurane diterpenoids through post-modified reactions of Baeyer-Villiger oxygenation and glycosylation.

Ent-kaurane diterpenoids are a big member of terpenoids with diverse structural features. More than 600 ent-kaurane diterpenoids have been isolated from different plants, especially from the genus Isodon (=Rabdosia)1–3. Recently, some novel ent-kaurane diterpenoids such as neoadenoloside A4, neolaxiflorins A and B5 with unique skeletons were reported, which led to a new wave of research hot about ent-kaurane diterpenoids. According to the structure features, Professor Fujita ever divided this member of diterpenoids into four groups: ent-kauranes, 6,7-seco-ent-kauranes, 8,9-seco-ent-kauranes, and others in 19844. Recently Professor Sun classified ent-kauranes into nine groups including C-20 non-oxygenated ones, C-20 oxygenated ones, 6,7-seco-ent-kauranes, 8,9-seco-ent-kauranes, 8,15-seco-ent-kauranes, 15,16-seco-ent-kauranes, 7,20-seco-ent-kauranes, ent-kaurane dimers, miscellaneous ent-kauranes1. In addition, there were also some reports about 2, 3-seco-ent-kauranes (Fig. 1)7. Although 3, 4-seco-sesquiterpenoids, and 3, 4-seco-triterpenoids were ever isolated from different sources, 3, 4-seco-ent-kaurane diterpenoids have never found in nature (Fig. 1).

Flickingeria fimbriata (B1.) Hawkes is a medicinal plant distributed in the south of China including Hainan, Guangxi, and Yunnan Provinces. Its whole herb has been used as tonic and antipyretic agents in the Minority Nation Li, in Hainan province. In addition, this medicinal plant (also named “You Gua Shi Hu”) was often used as the equivalent of Pale Ephemerantha ("Shi Hu") in the medicinal market. Previous chemical investigations of this plant obtained pimarane-type diterpenoids, several phenanthrenes, steroids, and bibenzyls7–12. During our ongoing search of new bioactive secondary metabolites from herbal medicines13–15, we initiated the chemical study of this medicinal plant collected from Hainan province. Ten new ent-kaurane diterpenoids fimbriatols A–J (1–10) including four 3, 4-seco-ent-kauranes (7–10) were isolated (Fig. 2). Herein we reported the isolation, structural elucidation, bioactive evaluation and postulated biosynthesis of 3, 4 ent-kaurane diterpenoids.

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Results and Discussion

Compounds 1–10 isolated from Flickingeria fimbriata (B1.) Hawkes.

Figure 1. Different types of seco-ent-kaurane diterpenoids.

Figure 2. Compounds 1–10 isolated from Flickingeria fimbriata (B1.) Hawkes.

Compound 1 had molecular formula C_{20}H_{32}O_{4} on the basis of its HRESIMS (m/z 337.2380 [M + H]^+, Δ − 0.1) indicating the presence of five degrees of unsaturation. Detailed analysis of the ^1H NMR, ^13C NMR, and HMOC data for 1 (Tables 1 and 2) revealed the presence of two methyls, ten methylene units (two oxygenated), three methines, and four quaternary carbons (one oxygenated), and one ketone group. These data accounted for all ^1H and ^13C resonances except for three exchangeable protons, and required 1 to be four cyclic systems. Interpretation of the ^1H–^1H COSY NMR data led to the identification of three isolated proton spin-systems corresponding to the C-1–C-2, C-5–C-6–C-7, and C-9–C-11–C-12–C-13–C-14 fragments of structure 1. The remaining connectivity was determined by HMBC correlations (Fig. 3). The correlations from CH_{3}-18 and OCH_{2}-19 to C-3, C-4, and C-5 confirmed that C-4 was connected with C-3, C-5, CH_{3}-18 and OCH_{2}-19, and correlations from CH_{2}-1 to C-3, and CH_{2}-15 with C-7, C-8, C-9, and C-14 confirmed that C-7, C-9, C-14 and C-15 all were connected with C-8. In the HMBC spectra, the distinct cross peaks from OCH_{2}-17 to C-13, C-15, and C-16 confirmed the
connectivity of C-16 with C-13, C-15 and C-17. Accounting for the molecular formula and chemical shift values of C-16, C-17 and C-19, it implied that these three carbons possessed hydroxyl groups, respectively. Thus the planar structure of 1 was characterized. SciFinder searching found a phyllocladane diterpenoid named 16,17,18-trihydroxyphyllocladane-3-one possessing the same planar structure as that of 14. Liu et al. ever summarized the diagnostic13 C NMR chemical shifts of selected phyllocladanes and ent-kauranes, which revealed that chemical shift values of C-13, C-14, C-15, C-16, C-17 and C-20 in these two member of diterpenoids changed regularly. From the13 C NMR chemical shifts of those carbons in compound 1, it implied that this structure should possess the skeleton of ent-kauranes but not that of phyllocladanes. This hypothesis was further confirmed by NOESY correlations and CD spectra. The correlations from H2-17 to H-15a, H-15a to H-11, H-9 to H-5, H-5 to CH3-18 implied the α-configuration of these protons, whereas the correlations of CH2-6 in compound 1 with H-11 and OCH2-19 established the β-configuration of these protons (Fig. 3), which established the relative configuration of compound 1 as kauranes. The NOESY correlation spectrum of compound 2 was same as that of compound 1 and the NOESY correlation spectrum of compound 3 was same as that of compound 2 by analysis of its HRESIMS. The NMR data especially the 2D NMR experiments suggested that the hydroxyl group was attached at C-9 not at C-6 found in 2 and 3. The NOESY correlation revealed the same relative configuration as those of 1–3 except for the 9-OH.

The HSIMS of 2 gave a pseudomolecular ion [M + Na]+ peak at m/z 375.2061 (Δ = +8.6), indicating the molecular formula of 2 as C30H30O4 (five degrees of unsaturation) with one more oxygen atom than that of 1. Analysis of NMR data revealed one additional oxygenated methine (δC 65.86; δH 4.29) present in NMR spectra of 2 compared with that of 1. The 1H–1H COSY and HMBC correlations confirmed that the CH2-6 in 2 was oxygenated to the corresponding oxymethine unit in 1. The relative configuration was established by analysis of coupling constant and NOESY correlations. The small coupling constant between H-5 and H-6 (J ≈ 10 Hz) together with the NOESY correlations from H-5, H-6 to CH3-18 implied the cis relationship of this two protons. The other NOESY correlations were same as those of 1 (Fig. 5). Thus the relative configuration of 2 was determined.

The molecular formula of 3 was same as that of 2 by analysis of its HRESIMS. The NMR data especially the 2D spectra revealed that 3 possessed the same planar structure as 2. The NOESY correlation spectrum of 3 was similar with that of 2, except that H-6 had correlations with H-19 and CH3-20, not correlation with CH3-18 in 2, leading to the trans configuration between H-5 and H-6, and this was also supported by the big coupling constant between H-5 and H-6 (J = 10.8) (Fig. 5).

Table 1.1H NMR data of compounds 1–6 recorded at 600 Hz. aRecorded in CD3OD, brecorded in DMSO-d6.

| Pos | 1a | 1b | 2 | 3 | 4 | 5 | 6 |
|-----|----|----|---|---|---|---|---|
| 1a  | 1b | 2.13, 1.33, m 2.05, m 1.32, m 1.81, m 1.47, m 2.31, m 1.77, m 2.22, ddd (3.6, 6.6, 13.2) 1.60, m 3.92, dd (1.2, 9.0) |
| 2   | 2.69, m 2.33, m 2.60, m 2.37, m 2.72, ddd (5.4, 11.4, 15.6) 2.12, ddd (5.4, 9.6, 15.6) 2.67, ddd (6.6, 11.4, 14.4) 2.19, ddd (4.8, 6.6, 14.4) |
| 5   | 1.42 m 1.33, s 1.64, d (10.8) 2.11, d (11.4) 1.46, d (10.8) 1.58, d (10.8) |
| 6   | 1.63, 1.53m 4.29, d (1.8) 3.80, dt (3.6, 10.8) 1.63, m 1.56, m 1.67, m 1.56, m 3.84, dt (3.6, 10.8) |
| 7αb | 1.66, 1.49m 1.84, m 1.74, m 1.67, m 1.48, m 1.88, d (5.4, 13.2) 1.36, m 1.73,m 1.56, m 1.81, d (4.2, 11.4) 1.61, m |
| 9   | 1.10 d (6.6) 1.20, br s 1.06, d (7.2) 1.30, s 1.33, d (7.2) |
| 11αb | 1.64 m 1.67, m 1.57, m 1.45, m 1.99, dd (7.2, 15.0) 1.30, m 3.93, d (7.2) 2.42, dd (7.2, 15.6) 1.68, m |
| 12  | 1.66, 1.56m 1.79, m 1.63, m 1.54, m 1.31, m 1.79, dd (7.2, 15.0) 1.61, m 2.04, m 1.87, m 1.59, m 1.48, m |
| 13  | 2.05 br.m 2.06, m 1.89, br s 2.06, br s 2.10, br s 2.03, br s |
| 14αb | 1.94, 1.64m 2.32, d (12.0) 1.83, d (12.0) 1.63, m 2.20, br d (12.0) 1.79, dd (12.0) 1.86, m 1.73, d (10.8) |
| 15αb | 1.54, 1.40m 1.61, d (14.4) 1.45, d (14.4) 1.46, m 1.32, d (13.8) 2.27, m 1.09, d (15.0) 2.14, brd (14.4) 1.34, d (14.4) 1.64, d (11.4) 1.46, d (14.4) |
| 17αb | 3.70, d (11.4) 3.60, d (11.4) 3.73, d (10.4) 3.63, d (10.4) 3.51, dd (10.8, 3.6) 3.42, dd (10.8, 4.8) 3.69, d (11.4) 3.58, d (11.4) 4.16, d (11.4) 3.76, d (11.4) 3.72, d (11.4) 3.62, d (11.4) |
| 18  | 1.13, s 1.12, s 1.22, s 1.15, s 1.17, s 1.30, s |
| 19αa | 3.97, d (11.4) 3.50, d (11.4) 4.13, d (10.4) 3.48, d (10.4) 3.97, d (10.8) 3.73, d (10.8) 4.02, d (11.4) 3.52, d (11.4) 3.97, d (11.4) 3.52, d (11.4) 1.30, s |
| 20  | 1.24s 1.49, m 0.82, s 1.36, s 1.17, s 0.93, s |
| 6-OH | 5.01, d (3.6) |
| 16-OH | 3.91, s |
| 17-OH | 4.35, dd (4.8, 3.6) |
| 19-OH | 5.11, brd (3.6) |
The HRESI MS (m/z 375.2134 [M + Na]+ Δ + 0.8) of compound 6 afforded the molecular formula as C_{20}H_{32}O_{5} same as that of compounds 2–5. The \(^1\)H NMR spectra displayed three methyl groups present in 6, implying that the 19-methyl was not oxidized. The \(^1\)H–\(^1\)H COSY and HMBC correlations confirmed that both C-1 and C-6 contained one hydroxyl group, respectively. The hydroxyl group for C-1 was determined to be \(\alpha\)-configuration on the basis of the NOESY correlations from H-1 to H-5 and H-9, and hydroxyl group at C-6 was established as
β-configuration on the basis of NOESY correlations of H-6 with CH-19 and CH-20. In addition, the large coupling constant between H-5 and H-6 (J ≈ 10.8 Hz) supported the trans relationship of these two protons.

Compounds 2–6 displayed the similar CD spectra with that of compound 1, which implied the same stereochemistry of 2–6 as that of 1 (Supporting Information).

The molecular formula of 7 was determined to be C_{32}H_{40}O_{12} on the basis of HRESI MS m/z 677.33214 ([M + Na]^+). The 1H and 13C NMR spectra of 7 revealed the significant difference compared with those of 1 (Table 3). The NMR spectra displayed a carboxylic group (δ_H 11.94; δ_C 174.7) and a double of exocyclic olefinic carbons (δ_H 4.83, 4.62; δ_C 147.1, 113.1) signals, whereas the keto carbonyl (C-3) and the oxymethylene (C-19) were disappeared. These differences implied that the carbon-bond of C-3/C-4 might be oxidized to shape the corresponding carboxylic group, and then dehydration reaction at C-19 formed the exocyclic double bond. The 2D NMR experiments especially the HMBC correlations from CH2-1 and CH2-2 to C-3 (δC 174.7) and from CH2-18 to C-4, C-5 and C-19 (exocyclic olefinic carbon) confirmed the above-mentioned postulation (Fig. 6). Thus the planar structure of 7 was determined. The relative configuration was established by NOESY correlations. Compound 7 possessed the same relative configuration as that of 1 depicted in Fig. 6.

Compound 8 possessed the molecular formula as C_{32}H_{38}O_{14} on the basis of HRESIMS (m/z 653.3179 [M]+, Δ + 2.4). Comparison of the NMR data of compound 8 and 7 revealed that the same structural fragments were found in 8 except for an additional oxethyl and an acetonide group present in 8 (Table 3). In addition, the carboxylic acid group, and the two hydroxyls at C-16 and C-17 were disappeared. This difference implied that the diol group at C-16/17 might react with acetone to form the acetonide product, and the carboxyl group might shape the oxethyl derivative with ethanol solvent, and this postulation was further supported by HMBC correlations. Because ethanol and acetone solvents were used in the process of isolation, compound 8 was postulated to be an artifact product from 7. We added 7 to acetone and ethanol solvent mixed with silica gel for a week. The mixture was then analyzed by TLC, which did produce compound 8 by comparison. This revealed that compound 8 should be the artifact product from 7.

The molecular formula of 9 was established as C_{31}H_{39}O_{13} by analysis of its HRESIMS [m/z 653.3179 (M + Na)]; 1H and 13C NMR spectra for 9 suggested the presence of the same structure fragment found in 7 and 8 except for two additional sugar moieties. The HMBC, 1H–1H COSY and HMBC correlations confirmed the two sugar moieties were glucose and apiose, respectively. The coupling constant of the anomic proton found in the glucose moiety (δ_H 4.37, 1H, d, J = 7.8 Hz) suggested a β-configuration glucose unit, whereas the coupling constant for the apiose anomic proton (δ_H 5.20, 1H, d, J = 3.0 Hz) and the chemical shift value for the anomeric carbon signal at δ_C 109.9 suggested the β-configuration apiose unit. The linkage of the sugar moieties was determined by HMBC correlations. The HMBC correlations from CH-2′-17 to C-1′-1 implied the connection of C-17 with C-1′ of 9. The linkage between C-2′ and C-1′′ by an ether bond was confirmed by HMBC correlations from H-1′′-2′-C-2′-C-2′-C-2′′-H-1″ by an oxygen atom. The linkage between C-2′ and C-1′′′ by an ether bond was confirmed by HMBC correlations from H-1″-2′-C-2′-C-2′′′-H-1″″. The chemical shifts of the glucose and apiose moiety were assigned through 1H–1H COSY, HMOC and HBMC spectra. Thus the planar structure of 9 was established. The relative configuration of 9 was same as that of 7 by analysis of the NOESY correlations.

The molecular formula of 10 was established as C_{32}H_{39}O_{13} by analysis of its HRESIMS [m/z 667.33214 (M + Na)]; 1H and 13C NMR spectra of 10 displayed resonances nearly identical to those of 9 except for an additional OMe group, suggesting that 10 was the oxymethyl derivative of 9. Analysis of HMBC data confirmed the above observations, and permitted the OMe unit to be connected with the carboxyl group (C-3). The relative configuration of 10 was same as those of 7 and 9 by analysis of its NOESY correlations. Due to the different groups found in compounds 7–10, the CD spectra of compounds 7–10 were different from those compounds 1–6 (supporting information), which could not determine the absolute configurations of 7–10 by CD spectra. From the biosynthetic view, these compounds were originated from same diterpene biogenetic pathway. Thus compounds 7–10 were postulated to possess the same absolute configurations as those 1–6. The configurations of two sugar moieties were determined to be D-glucose and D-apiose by hydrolysis and GC-MS methods.

Protein phosphorylation as posttranslational modification plays a vital role to regulate cell activities. In cells, the protein kinases and phosphatases control the protein phosphorylation level. Dual-specificity phosphatase 26 (DUSP26) is a heterogeneous group of protein phosphatases, and can dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues. Due to the fact that DUSP26 is located at 8p, a chromosomal region that has been shown to be amplified in anaplastic thyroid cancer (ATC), this enzyme has emerged as a potential target for the treatment of human cancers by dephosphorylating p38 MAPK, thereby inhibiting p38-mediated cellular signaling.
| Pos | δ H (ppm) | J H-H (Hz) | δ C (ppm) | J C-C (ppm) |
|-----|-----------|------------|-----------|------------|
| 1   | 33.8 CH₃  | 1.56, m 1.43, m | 33.8 CH₃  | 1.64, m    |
| 2a  | 28.1 CH₂  | 2.17, dd (4.2, 12.6, 15.6) 1.99, dd (6.0, 12.0) | 28.8 CH₂  | 2.26, dd (4.8, 10.8, 16.2) 2.11, dd (6.6, 11.4, 16.2) |
| 3   | 174.7 C   | 174.0 C    | 177.7 C   | 176.4 C    |
| 4   | 147.1 C   | 147.3 C    | 148.2 C   | 148.8 C    |
| 5   | 49.2 CH   | 1.96, dd (2.4, 12.0) | 50.2 CH   | 1.98, dd (2.4, 12.6) |
| 6   | 26.0 CH   | 1.71, m 1.31, m | 26.3 CH   | 1.76, m 1.42, m |
| 7   | 40.0 CH   | 1.42, m    | 39.8 CH   | 1.52, m 1.43, m |
| 8   | 43.5 C    | 44.1 C     | 44.1 C    | 45.4 C     |
| 9   | 46.0 CH   | 1.11, d (7.8) | 46.3 CH   | 1.11, d (8.4) |
| 10  | 40.2 C    | 40.8 C     | 41.0 C    | 42.0 C     |
| 11  | 17.8 CH   | 1.53, m 1.38, m | 19.0 CH   | 1.53, m 1.30, m |
| 12  | 25.5 CH   | 1.54, m 1.36, m | 26.87 CH | 1.50, m |
| 13  | 44.4 CH   | 1.90, brd (3.0) | 45.5 CH   | 2.16, m |
| 14a| 36.5 CH   | 1.74, m 1.57, d (10.8) | 38.1 CH₁ | 1.93, dd (0.6, 10.8) 1.49, m |
| 15a| 52.8 CH   | 1.45, m 1.26, d (14.4) | 56.6 CH   | 1.87, d (14.4) 1.67, d (14.4) |
| 16  | 80.2 C    | 88.9 C     | 79.8 C    | 82.0 C     |
| 17a| 65.1 CH₁  | 3.51, d (6.0, 10.8) 3.40, d (5.4, 10.8) | 69.9 CH₂ | 4.05, d (8.4) 3.91, d (8.4) |
| 18  | 23.1 CH₁  | 1.69, s | 23.3 CH₂ | 1.73, s |
| 19  | 113.1 CH₁ | 4.83, s 4.62, s | 113.6 CH₂ | 4.86, s 4.64, s |
| 20  | 21.7 CH₁  | 0.92, s   | 21.8 CH₁ | 0.98, s |
| 1’16-OH | 4.33, t (5.4) | 60.3 CH₂ | 4.10, q (7.2) |
| 2’17-OH | 3.86, s | 14.2, CH₃ | 1.24, t (7.2) |
| 3’COOH | 11.94, s | 108.4, C  | 76.8, CH   | 3.32, m |
| 4’ | 26.86, CH₃ | 1.38 s | 70.6, CH | 3.10, m |
| 5’ | 26.80, CH₃ | 1.35 s | 77.1, CH | 3.10, m |
| 6’ | 61.4, CH₂ | 3.45, d (11.4) 3.65, d (11.4) | 62.7, CH₂ | 3.88, d (11.4) 3.67, d (6.0, 11.4) |
| 7’ | 109.7 CH₁ | 5.18, d (3.0) | 112.3 CH₁ | 5.20, d (3.0) |
| 8’ | 76.3, CH | 3.75, d (3.0) | 77.4, CH | 3.95, d (3.0) |
| 9’ | 79.0, C | 79.7, C |
| 10’ | 73.4, CH₂ | 3.95, d (9.0) 3.52, d (9.0) | 74.6, CH₂ | 4.07, d (9.6) 3.73, d (9.6) |
| 11’ | 64.2, CH₂ | 3.39, d (11.4) 3.34, d (11.4) | 65.0, CH₂ | 3.57, d (11.4) 3.56, d (11.4) |

Table 3. 1H NMR and 13C NMR spectroscopic data of 7 (in DMSO-d₆), 8 (in CDCl₃), 9 (in DMSO-d₆) and 10 (in CD₃OD).

apoptosis[^21]. Only one compound, 8-hydroxy-7-(6-sulfonaphthalen-2-yl)diazenyl-quinoline-5-sulfonic acid (NSC-87877), had been found as a DUSP26 inhibitor with the against DUSP26 IC₅₀ value of 18.6 µM[^19], however, up to date no research was reported about natural products inhibiting against DUSP26. Thus compound 1 was tested inhibitory activities against DUSP26, and displayed biological activity with inhibitory ratio at 48.5% compared with 81.6% of the positive compound NSC-87877. Though the bioactive result of compound 1 was not good as the positive compound, the skeleton feature of 1 is completely different from that of the positive compound NSC-87877, which might imply their possibly different site of action.

In addition, compounds 1–10 were also tested against the activities of several plant pathogens, acetylcholinesterase, xanthinoxidase (XOD) and cytotoxic activities without any effects.

Up to date, more than 600 ent-kaurane diterpenoids with diverse structurally features have been isolated from different plant[^13]. Compounds 1–6 are different from all known analogues by possessing highly oxidized sites, whereas 7–10 contain the 3, 4-seco-ent-kaurane skeleton, which is the first report in all ent-kaurane diterpenoids. Mono-glycosylated ent-kaurane diterpenoids are ever isolated from different resource, whereas 9 and 10 contained a disadvantage to C-17 with the unusual linkage at C-2′ to C-1′, which was the first report in all known ent-kaurane diterpenoids. Recently, Hu et al. confirmed that a flavin-dependent monoxygenase catalyzed a keto group to the corresponding ester moiety by Baeyer-Villiger reaction in *Aspergillus clavatus[^21]*. Thus we postulated that compounds 7–10 might be biosynthesized from ent-kaurane diterpenoids firstly through Baeyer-Villiger oxidation reaction to cleave the carbon
bond between C-3 and C-4, and then through hydration, dehydration and glycosylation reactions to form 9 and 10 by post-modification (Fig. 7). Considering the highly oxidized sites in compounds 1–10, it implies that there exist diverse P450 oxidases or flavin-dependent monoxygenases in this plant, which could produce different ent-kaurane diterpenoids analogues, and thus further chemical investigation of this medicinal plant should be in progress to mine more new ent-kaurane diterpenoids. In conclusion, ten new ent-kaurane diterpenoids possessing highly oxidized sites were isolated from *Flickingeria fimbriata* (B1.) Hawkes. Our result further diversifies the structural feature of ent-kaurane diterpenoids.

**Methods**

**General experimental procedures.** Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on Beckman Coulter DU 800 spectrometer. IR data were recorded using a Shimadzu FTIR-8400S spectrophotometer. 1H and 13C NMR data were acquired with a Bruker 600 spectrometer using solvent signals (CDCl3, δH 7.26/δC 77.6, CD3OD, δH 3.31/δC 49.9, DMSO-d6, δH 2.49/δC 39.5) as references. The HMOC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. HRESIMS data were acquired using a LTQ Orbitrap XL mass spectrometer.

**Plant material.** The plant of *Flickingeria fimbriata* (B1.) Hawkes was collected from Changjiang, Hainan Province, People's Republic of China, in August 2008. The sample was identified by Mr. Rong-tao Li (Hainan Branch of Institute of Medicinal Plant Development), and a voucher specimen (FF2008-1) has been deposited in the herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing.

**Extraction and isolation.** The air-dried and smashed stems of *F. fimbriata* were extracted with 95% EtOH to afford a crude extract after evaporation under vacuum. The extract was suspended in H2O and then partitioned sequentially with petroleum ether, CH2Cl2, EtOAc, and n-BuOH, successively. The CH2Cl2 and EtOAc extracts were subjected to column chromatography over silica gel to obtain 10 new ent-kaurane diterpenoids named fimbriatols A–J (1–10), which were characterized by HRESIMS, NMR and X-ray diffraction experiments.

The air-dried and smashed stems of *F. fimbriata* (3.5 kg) were extracted with 95% EtOH (3 × 14 L) to afford a crude extract (330 g) after evaporation under vacuum. The extract was suspended in H2O and then partitioned sequentially with petroleum ether (3 × 3.0 L), CH2Cl2 (3 × 3.0 L), EtOAc (3 × 3.0 L), and n-BuOH (3 × 3.0 L), successively. The CH2Cl2 extract (80.0 g) was subjected to column chromatography over silica gel (80–100 mesh), eluted with petroleum ether-acetone (10:1) as the mobile phase to yield eight fractions (C1–C8). E4 (3.2 g) was subjected to column chromatography over silica gel (100–200 mesh) repeatedly to obtain 8 (21 mg). E5 (5.0 mg) was subjected to column chromatography over silica gel (100–200 mesh), Sephadex LH-20, ODS and HW-40C (ethanol: H2O = 1:1) to afford compounds 2 (2.0 mg), 3 (2.0 mg), and 5 (10.0 mg), respectively. The EtOAc extract (10.0 g) was subjected to column chromatography over silica gel (100–200 mesh), Sephadex LH-20, ODS and HW-40C (EtOH: CH2Cl2, CH2Cl2, CH2Cl2: CH2Cl2: CH2Cl2: CH2Cl2, 1:1:1:1) to yield eight fractions (E1–E7). E6 (1.0 g) was subjected to column chromatography over silica gel (100–200 mesh), eluted with CH2Cl2:CH2Cl2:CH2Cl2 (50:1:3:1) to yield eight fractions (E1–E7). E7 (18.0 mg) was subjected to column chromatography over silica gel (100–200 mesh), eluted with ether-acetone (3:2) and then recrystallized to afford compounds 1 (3.0 g) and 7 (15 mg), respectively. E7 (18.0 g) eluted with petroleum ether-acetone (1:1) was subjected to column chromatography over silica gel (100–200 mesh), Sephadex LH-20, ODS and HW-40C to 10 (14.6 mg). E7 (1.5 g) was subjected to column chromatography over silica gel (100–200 mesh), then Sephadex LH-20, ODS and HW-40C to 9 (73.0 mg). E7 (6.0 mg) eluted with petroleum ether-acetone (1:1) was subjected to column chromatography over silica gel (100–200 mesh), eluted with ether-acetone (7:3–0:1) and Sephadex LH 20 column chromatography to obtain 4 (5.0 mg) and 6 (7.5 mg), respectively. Fimbriatol A (4R, 16R)-16, 17, 19- trihydroxy-ent-kaur-3-one (1): white crystal; mp 194–196°C; [α]20D +20.0 (c 0.06, MeOH); UV (MeOH) λmax (log ε) 205 (0.5), 282.5 (0.027) nm; IR (KBr) νmax 3372 (OH), 2928 (CH), 1693 (C=O) cm−1; 1H and 13C NMR see Tables 1 and 2; HRESI-MS m/z 337.2380 [M + H]+ (calcd for C20H33O4 Na, 337.2379).

Fimbriatol B (4R, 6S, 16R)-6, 16, 17, 19- tetrahydroxy-ent-kaur-3-one (2): white crystal; mp 220–222°C; [α]20D −10.0(c 0.05, MeOH); UV (MeOH) λmax (log ε) 210 (0.16), 292.5 (0.018) nm; IR (KBr) νmax 3200 (OH), 2922 (CH), 1696 (C=O) cm−1; 1H and 13C NMR see Tables 1 and 2; HRESI-MS m/z 375.2061 [M + Na]+ (calcd for C20H32O5Na, 375.2142).
Fimbriatol C (4R, 6R, 16R)-6, 16, 17, 19- tetrahydroxy-ent-kaur-3-one (3): white crystal; mp. 226–228 °C; \([\alpha]_D^{20} = -38.0 (c 0.075, MeOH); UV (CH₃OH) \lambda_{max} (log e) 210 (0.30) nm; IR (KBr) \nu_{max} 3381 (OH), 2931(CH), 1696 (C=O) cm⁻¹; ^1H and ^13C NMR see Table 1 and 2; HRESI MS (+) m/z 375.2164 [M + Na]⁺ (calcd for C₂₀H₃₂O₅Na,375.2142).

Fimbriatol D (4R, 16R)-9, 16, 17, 19- tetrahydroxy-ent-kaur-3-one (4): white crystal; mp. 116–118 °C; \([\alpha]_D^{20} = -11.4 (c 0.07, MeOH); UV (CH₃OH) \lambda_{max} (log e) 212 (0.26) nm; IR (KBr) \nu_{max} 3367 (OH), 2945(CH), 1685 (C=O) cm⁻¹; ^1H and ^13C NMR see Tables 1 and 2; HRESIMS (+) m/z 375.2169 [M + Na]⁺ (calcd for C₂₀H₃₂O₅Na,375.2142).

Fimbriatol E (4R, 11S, 16R)-11, 16, 17, 19- tetrahydroxy-ent-kaur-3-one (5): white crystal; mp. 202–204 oC; \([\alpha]_D^{20} = -16.7 (c 0.06,MeOH); UV (CH₃OH) \lambda_{max} (log e) 212 (0.26), 282.5 (0.015) nm; IR (KBr) \nu_{max} 3382 (OH), 2931 (CH), 1694 (C=O) cm⁻¹; ^1H and ^13C NMR see Tables 1 and 2; HRESIMS (+) m/z 375.2166 [M + Na]⁺ (calcd for C₂₀H₃₂O₅Na,375.2142).

Fimbriatol F (15S, 6R, 16R)-1, 6, 16, 17- tetrahydroxy-ent-kaur-3-one (6): amorphous powder; mp. 284–286 °C; \([\alpha]_D^{20} = -59.1 (c 0.055,MeOH); UV (CH₃OH) \lambda_{max} (log e) 206 (0.27), 285 (0.022) nm; IR (KBr) \nu_{max} 3378 (OH), 2928 (CH), 1708 (C=O) cm⁻¹; ^1H and ^13C NMR see Tables 1 and 2; HRESIMS (+) m/z 375.2134 [M + Na]⁺ (calcd for C₂₀H₃₂O₅Na,375.2142).

Fimbriatol G (7): white crystal; mp 236–238°C; \([\alpha]_D^{20} = -68.3 (c 0.01, MeOH); UV (CH₃OH) \lambda_{max} (log e) 204 (1.48), 237 (0.021) nm; IR (KBr) \nu_{max} 3347 (OH), 2925(CH), 1706 (C=O) cm⁻¹; ^1H and ^13C NMR data see Table 3; HRESI-MS m/z 359.2198 [M + Na]⁺ (calcd for C₁₇H₂₅O₃Na,359.2198).

Fimbriatol H (8): white crystal; mp 200-202°C; \([\alpha]_D^{20} = -19.5 (c 0.02, MeOH); UV (CH₃OH) \lambda_{max} (log e) 205 (5.0) nm; IR (KBr) \nu_{max} 2926(CH), 1734 (C=O) cm⁻¹; ^1H and ^13C NMR see Table 3; HREIMS (m/z 404.2903 [M]⁺ (calcd for C₂₅H₃₉O₅Na, 404.2927).

Fimbriatol I (9): brown oil; \([\alpha]_D^{20} = -80 (c 0.01, MeOH); UV (CH₃OH) \lambda_{max} (log e) 202 (1.2) nm; IR (KBr) \nu_{max} 3401 (OH), 2929 (CH), 1705 (C=O) cm⁻¹; ^1H and ^13C NMR see Table 3; HRESI-MS m/z 653.3179 (M + Na)⁺ (calcd for C₁₇H₂₄O₄Na,653.3149).

Fimbriatol J (10): brown oil; \([\alpha]_D^{20} = -66 (c 0.01, MeOH); UV (CH₃OH) \lambda_{max} (log e) 202 (1.263) nm; IR (KBr) \nu_{max} 3376 (OH), 2929 (CH), 1730 (C=O) cm⁻¹; ^1H and ^13C NMR see Table 3; HRESI-MS m/z 667.3328 (M + Na)⁺ (calcd for C₁₇H₂₅O₅Na, 667.3307).

Hydrolysis reaction and GC-MS analysis. Compounds 9 and 10 (1mg each) were put in 1 N HCl (0.25 mL) and stirred at 80°C for 4h. Then after cooling, the solution was dried by blowing air²⁸. The residue was dissolved in 1-(trimethylsilyl)-imidazole and pyridine (1 mL). The solution was stirred at 60°C for 5 min. After drying the solution with a stream of air, the residue was separated by water and CH₂Cl₂ (1 mL, v/v = 1:1). The CH₂Cl₂ layer was analyzed by GC (Agilent technologies 7890B system) using an capillary HP-5 column (30 m x 250 μm) at 25 μm. Temperature was maintained at 50°C for 1 min, then raised to 100°C at the rate of 5°C/min (5 min), and raised to 265°C at the rate of 5°C/min (11 min). Peaks of the hydrolysate of 9 and 10 were detected at 15.25, 15.32, 16.49 min, and 15.25, 15.32, 16.49 min, respectively. Peaks of D-apiose, were 15.27, 16.49 min, and D- and L-glucose were 15.25, 16.49 min, and D- and L-glucose were 15.25, 16.49 min, and D-$\alpha$-apiose were 15.27, 16.49 min.

X-ray crystallographic analysis of fimbriatolone (1). Crystallization from petroleum MeOH: H₂O (10: 1) yielded colorless prisms of 1. A crystal (0.08 × 0.14 × 0.52mm) was separated from the sample and mounted on a glass fiber, and data were acquired with a Rigaku MicroMax 002 + diffractometer with graphite-monochromated Mo Kα radiation and a graphite monochromator. Structure analysis was made using the SHELXL97 program. Crystallographic data (excluding structure factors) for 1 in this paper have been deposited with the Cambridge Crystallographic Data Centre (deposition number: CCDC 1063873). Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: 44 12 23363033 or...
DUSP26 was subcloned from human DUSP26 cDNA (Invitrogen, Carlsbad, CA, USA) into the expression vector pGEX-4T-2 as a fusion with a N-terminal GST-tag and a thrombin protease cleavage site. For proteins expression, *Escherichia coli* cells growing in 1 L LB media at OD600 of near 0.8 were induced with 0.5 mM IPTG at 16 °C for 16 h. Centrifuged culture pellets were extracted by sonication in PBS buffer with 100 mM PMSF. Extracts were clarified by centrifugation 13,000 rpm for 30 min at 4 °C to yield the soluble extract. GST-tagged proteins present in the soluble extracts were purified by GST-affinity chromatography and dialyzed overnight against buffer containing 50 mM Tris–HCl (pH 7.5), and 0.1 M NaCl.

**Assays of DUSP26 inhibition.**  
In vitro phosphatase assay used for determining whether compounds are DUSP26 inhibitors was established and optimized based on methods described previously. The activity of DUSP26 was measured using the substrate p-nitrophenyl phosphate (pNPP; Sigma, St. Louis, MO) at the concentration using a 96-well microtiter plate. NSC-87877 (positive inhibitor; Calbiochem, San Diego, CA) and pNPP were solubilized in ddH2O. The compound at a 96-well microtiter plate. NSC-87877 (positive inhibitor; Calbiochem, San Diego, CA) and pNPP were solubilized in ddH2O. The compound I was solubilized in HPLC grade DMSO with concentration at 0.022 μg/mL. All reactions were performed at a final concentration of 1% DMSO, while the enzyme activity was not affected by this concentration of DMSO.

The purified DUSP26 (10 nM), and a candidate inhibitor were incubated in the reaction mixture containing 50 mM Tris–HCl (pH 7.5), 1% DMSO, for 15 min at 37 °C. Reactions were initiated by addition of pNPP and the incubation time was 30 min at 37 °C. This enzymatic reaction was stopped with the addition of 0.2 M NaOH. The phosphatase activities were then monitored by measuring the absorbance changes due to the hydrolysis of the substrate at 405 nM. The activities of DUSP26 with and without candidate inhibitors were represented as Ai and A0. The value of Ai/A0 × 100% was the residual activity (R.A.) of DUSP26. All experiments were performed in triplicate.

**References**

1. Sun, H. D., Huang, S. X. Q. & Han, B. Diterpenoids from *Isodon* and their biological activities, Nat. Prod. Rep. 23, 673–698 (2006).
2. Yang, Y. L. et al. New ent-kaurane diterpenoids with anti-platelet aggregation activity from *Amomum squamosum*. J. Nat. Prod. 65, 1462–1467 (2002).
3. Han, L. et al. New diterpenoids from the marine mangrove *Bruguiera gymnorrhiza*. J. Nat. Prod. 67, 1620–1623 (2004).
4. Zhao, W. W. et al. Neoadenoloside A, a highly functionalized diterpene C-glycoside from *Isodon adenolomus*. Chem. Commun. 38, 7723–7725 (2012).
5. Wang, W. G. et al. New bicyclo[3.1.0]hexane unit ent-kaurane diterpene and its seco-derivative from *Isodon eriocalyx var. laxiflora*. Org. Lett. 14, 302–305 (2012).
6. Fujita, E. & Node, M. In: *Progress in the chemistry of Organic Natural Products*, (eds Herz, W., Grisebach, H., Kirby, G. W. & Tamm, Ch.) Ch. 46, 77–157 (Springer, 1984).
7. Tschiritzis, F. & Jakupovic, J. Diterpenes and other constituents from *Relhania species*. Phytochemistry 29, 3173–3187 (1990).
8. Ma, G. X. et al. A 19-carbon pimarane-type diterpenoid from *Ephemerantha fimbriata*. Pharm. Biol. 36, 66–68 (1998).
9. Chen, J. L. et al. Bioactive norditerpenoids from *Flickingeria fimbriata*. RSC Adv. 4, 144147–144156 (2014).
10. Chen, J. L. et al. Norditerpenoids from *Flickingeria fimbriata* and their inhibitory activities on nitric oxide and tumor necrosis factor-α production in mouse macrophages. Molecules 19, 5863–5875 (2014).
11. Chen, H. Y. et al. Antioxidant principles from *Ephemerantha ionchophylla*. J. Nat. Prod. 62, 1225–1227 (1999).
12. Chen, C. C., Huang Y. L. & Tang, C. M. Antiplatelet aggregation principles from *Ephemerantha ionchophylla*. Planta Med. 66, 372–373 (2000).
13. Yu, Y. M. et al. Lactones from *Angleotia caudiformis*. J. Nat. Prod. 72, 921–924 (2009).
14. Hou, G. A. et al. Lactonecembranoids from *Croton leuca* var. *Croton junci*. J. Nat. Prod. 73, 792–795 (2010).
15. Yu, L. L. et al. Gusanlflowerosides A–D, potential tyrosinase inhibitors from *Angeliangesia gusanlung*. J. Nat. Prod. 74, 1009–1014 (2011).
16. Shao, Y., Hu, H. L., Sim, K. Y. & Goh, S. H. Lignanoids and diterpenoids from *Callicarpa furcariae*. Helv. Chim. Acta 89, 64–72 (2006).
17. Liu, G., Müller, R. & Rüedi, P. Chemical transformations of phyllocladane (=13β-kaurane) diterpenoids. Helv. Chim. Acta 86, 420–438 (2003).
18. Mariano, S. D. et al. Bioactive asterosaponins from the Starfish *Luidia quinaria* and *Pelaster cassiope*. Isolation and structure characterization by two-dimensional NMR spectroscopy. J. Nat. Prod. 66, 515–519 (2003).
19. Song, M. et al. NSC-87877, inhibitor of SHP-1/2 PTPs, inhibits dual-specificity phosphatase 26 (DUSP26). Biochem. Biophys. Res. Com. 381, 491–495 (2009).
20. Patterson, K. L., Brummer, T., O’Brien, P. M. & R. J. Daly, Dual-specificity phosphatases: critical regulators with diverse cellular targets. Biochem. J. 418, 475–489 (2009).
21. Hu, Y. C. et al. A Carbonate-forming Baeyer-Villiger monooxygenase. Nat. Chem. Biol. 10, 552–554 (2014).
22. Yu, W., Imoto, I., Inoue, J., Onda, M., Emi, M. & Inazawa, J. A novel amplification target, DUSP26, promotes anaplastic thyroid cancer cell growth by inhibiting p38 MAPK activity. Oncogene 26, 1178–1187 (2006).
23. Won, E. Y. et al. High-resolution crystal structure of the catalytic domain of human dual-specificity phosphatase 26. Acta Cryst. D69, 1160–1170 (2013).

**Acknowledgements**

This work was financially supported by the Chinese National S&T Special Project on Major New Drug Innovation (2013ZX09508104), Program for Innovative Research Team in IMPLAD (PIRTI, IT1305), the Fundamental
Research Funds for the Central Scientific Research Institutes for Public Welfare, National Natural and Science Foundation of China (No. 81273432), and Program for New Century Excellent Talents in University.

**Author Contributions**
Z.Z. designed the experiment, G.D., J.F., J.W., Y.X., R.L., N.G. and Y.L. did the experiment; G.D., C.Y. and Z.Z. wrote the manuscript.

**Additional Information**
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Ding, G. et al. Fimbriatols A-J, Highly Oxidized ent-Kaurane Diterpenoids from Traditional Chinese Plant *Flickingeria fimbriata* (B1.) Hawkes. *Sci. Rep.* 6, 30560; doi: 10.1038/srep30560 (2016).

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