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New *ent*-kaurane diterpenoid acids from *Nouelia insignis* Franch and their anti-inflammatory activity†

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Eleven undescribed *ent*-kaurane-type diterpenoid acids, namely noueinsiancins A–K (1–11), together with sixteen related known analogs (12–27) were isolated from *Nouelia insignis* Franch. The chemical structures and absolute configurations of the new compounds were confirmed by the extensive spectroscopic data, electronic circular dichroism (ECD) data analysis and single crystal X-ray diffraction. Additionally, the anti-inflammatory assay was applied to estimate the nitric oxide (NO) inhibitory activities of all compounds by using lipopolysaccharide (LPS)-induced RAW 264.7 cells *in vitro*. The results revealed that 4–7 and 13–17 significantly inhibited NO production at the concentrations of 2.5 μ M, 5.0 μ M and 10.0 μ M. Meanwhile, compounds 6 and 7 were found to down-regulate the protein expression levels of IL-6 and TNF- α in RAW 264.7 cells induced by LPS in a dose-dependent manner. In conclusion, these findings provided the reference values for exploring the new chemicals with biological activities from this genus.

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1. Introduction

Nouelia insignis Franch belonging to the genus *Nouelia* is one of the infrequent woody plants from the Asteraceae family. It is widely distributed at an altitude of 1000–2500 meters in the mountainous areas of the Yunnan and Sichuan Provinces, especially in Panzhihua City in China. It is an endemic and endangered woody remnant species in our country due to the limited distribution and human deforestation.^{1–4} Meanwhile, the roots and leaves of *Nouelia insignis* Franch have been widely applied in traditional Chinese medicine (TCM), and have a long history of use to treat acute icteric hepatitis, chronic hepatitis, bacterial infections, inflammation, *etc.*^{5,6} At present, we find that there are few studies on the chemical ingredients of this plant based on the previous literature and querying a database (CA). Consequently, only a few biological activity studies were reported. In this manuscript, our phytochemical investigation of the MeOH extract of *Nouelia insignis* Franch led to the isolation and the identification of 27 *ent*-kaurane diterpenoid acids, including eleven new ones (1–11), together with sixteen related known analogs (12–27). Herein, the details of the

isolation, structural elucidation and anti-inflammatory activities of the new compounds are reported (Fig. 1).

2. Experimental

2.1 General experimental procedure

Column chromatography (CC): silica gel H (10–40 μ m) and silica gel (200–300 mesh) (Marine Chemical Factory, Qingdao, P. R. China). MCI gel CHP-20P: (Daiso, Co., Japan) and RP-C18 gel (40–63 μ m; Daiso, Co., Japan) were used for column chromatography. Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ, USA); TLC: silica gel plates, visualization by spraying with 10% H₂SO₄ in EtOH and Dragendorff's reagent. Semi-preparative HPLC: Agilent 1260 series (Agilent Technologies, US) with a Zorbax SB-C₁₈ (5 μ m, 9.4 mm \times 25 cm) column. NMR spectra: Bruker Avance III-500 spectrometer (Bruker, Switzerland). MS: Agilent MSD-Trap-XCT (for ESI) and Agilent-6520 Q-TOF mass spectrometer (for HR-ESI). IR: Thermo Scientific Nicolet 6700 (Thermo Scientific, US). UV spectra: Agilent 1260 series DAD detector (Agilent Technologies, US). CD spectrum: Brighttime Chirascan (Applied Photophysics Ltd, UK). Optical rotation: Rudolph Autopo V (Rudolph Research Analytical, Hackettstown, NJ).

2.2 Plant material

Nouelia insignis Franch were collected from Sichun province of China in August 2020. It was identified by Prof. Bao-kang Huang (Department of Pharmacognosy, Second Military Medical University, Shanghai, China). A voucher specimen (no. 202008-

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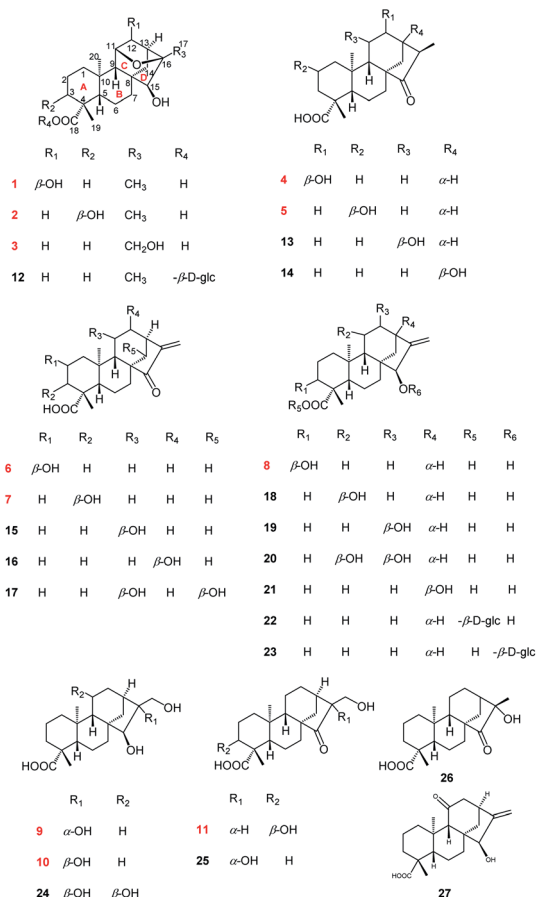


Fig. 1 Chemical structures of 1–27.

VS) is deposited in the department of pharmacognosy, Second Military Medical University.

2.3 Extraction and isolation

The dried and crushed branch of *Nouelia insignis* Franch (20.0 kg) were ultrasonically extracted with 95% methanol overnight at room temperature (20 L \times 3). After remove of solvent, the methanol extract (550.5 g) was extracted with water, petroleum ether (PE) and ethyl acetate (EtOAc) (3 times with 5 L each). The EtOAc extract (124.3 g) was segmented by silica gel H (10–40 μ m) column chromatography eluting with petroleum ether–ethyl acetate (PE/EtOAc, 50 : 1 to 0 : 1 v/v) to afford 7 fractions (Fr. 1–7). Fraction 4 (21.6 g) was separated by MCI column chromatography (MeOH/H₂O, 10 : 90 to 100 : 0 v/v) to provide 5 sub-fractions (Fr. 4.1–4.5). Then, Fr. 4.3 was separated by ODS (CH₃CN/H₂O, 10 : 90 to 40 : 60 v/v) to afford 6 sub-fractions (Fr. 4.3.1–4.3.6). Fr. 4.3.2 and Fr. 4.3.4 were purified by semi-preparative RP-C₁₈ HPLC (CH₃OH/H₂O, 63 : 37 v/v 1.0 mL min^{−1}) to give compounds 4 (12.3 mg), 5 (10.2 mg), 13 (9.4 mg), 14 (13.6 mg) and 8 (9.5 mg), 18 (10.6 mg), 19 (9.2 mg), 21 (8.2 mg). Fr. 4.5 was also separated by ODS (CH₃OH/H₂O, 10 : 90 to 40 : 60 v/v) to afford compounds 22 (15.2 mg), 23 (9.4 mg). Fraction 5 was initially separated by Sephadex LH-20 CC eluting with CH₃OH/H₂O (55 : 45) to yield 5 sub-fractions (Fr. 5.1–5.5).

Compounds 1 (14.3 mg) and 2 (10.3 mg) were obtained from Fr. 5.2 by ODS column chromatography (CH₃OH/H₂O, 30 : 70 to 55 : 45 v/v). Fr. 5.3–5.4 were purified by ODS column chromatography (CH₃OH/H₂O, 30 : 70 to 80 : 20 v/v) and further purified by semi-preparative RP-C₁₈ HPLC (CH₃OH/H₂O, 65 : 35 v/v 1.0 mL min^{−1}) to afford compounds 3 (13.3 mg), 12 (9.8 mg) and 9 (8.3 mg), 10 (11.8 mg), 24 (14.1 mg), 25 (9.7 mg). Fraction 6 was separated on a Sephadex LH-20 CC eluted with MeOH/H₂O (60 : 40, v/v) to yield 7 subfractions (Fr. 6.1–6.7), compound 11 (8.3 mg) was obtained from Fr. 6.1 through the ODS column chromatography (CH₃OH/H₂O, 30 : 70 to 55 : 45 v/v). Meanwhile, purifications of Fr. 6.3 and 6.4 by RP-HPLC (CH₃OH/H₂O, 60 : 40 v/v 1.0 mL min^{−1}) to yielded compounds 6 (9.8 mg), 7 (13.1 mg) and 17 (9.1 mg). Fr. 6.5 was separated with ODS column chromatography (CH₃OH/H₂O, 10 : 90 to 50 : 50 v/v) and further purified by semi-preparative RP-C₁₈ HPLC (CH₃OH/H₂O, 60 : 34 v/v 1.0 mL min^{−1}) to get compounds 15 (8.8 mg), 16 (12.7 mg) and 26 (13.3 mg). Fraction 7 was separated with ODS column chromatography (CH₃OH/H₂O, 10 : 90 to 40 : 60 v/v) and semi-preparative RP-C₁₈ HPLC (CH₃CN/H₂O, 62 : 38 v/v 1.0 mL min^{−1}) to give compounds 20 (9.3 mg) and 27 (8.6 mg). Above all, the whole compounds were obtained (Fig. 1).

2.4 Compound characterizations of 1–11

Noueinsiancin A (1). Colorless needle crystals; $[\alpha]_D^{25}$ −71.4 (*c* 0.06, CH₃OH); IR (KBr) ν_{\max} 3592, 3411, 2974, 2913, 2854, 1677, 1470, 1453, 1197, 1094, 1023, 982, 797, cm^{−1}; ¹H- and ¹³C-NMR data (500 MHz/125 MHz), see Tables 2 and 1; positive HRESIMS *m/z* 373.1962 ([*M* + Na]⁺, calcd for C₂₀H₃₀O₅Na, 373.1985). Crystal data for 1, size 0.20 \times 0.14 \times 0.09 mm³, orthorhombic, space group *P*₂12₁2₁, *a* = 7.4300 (3) Å, *b* = 12.6486 (5) Å, *c* = 21.6486 (13) Å, *V* = 1952.27 (13) Å³, *T* = 293.0 K, *Z* = 4, *D* = 1.315 Mg m^{−3}, λ (CuK α) = 1.54178 Å, *F*(000) = 840, reflections collected/unique 15 527/3520 [*R*(int) = 0.0500], *h* (−8/8), *k* (−14/14), *l* (−26/24), final *R* indices *R*₁ = 0.0389 and *wR*₂ = 0.1043 (*I* > 2 σ (*I*)), *R*₁ = 0.0404 and *wR*₂ = 0.1057 (all data), GOF = 1.093, largest diff. peak/hole, 0.387/−0.236 e Å^{−3}. Absolute structure parameter: 0.04(8). CCDC: 2127069.†

Noueinsiancin B (2). Colorless needle crystals; $[\alpha]_D^{25}$ −44.8 (*c* 0.07, CH₃OH); IR (KBr) ν_{\max} 3593, 3413, 2975, 2911, 1677, 1471, 1445, 1265, 1180, 1394, 1093, 1023, 935, 797 cm^{−1}; ¹H- and ¹³C-NMR data (500 MHz/125 MHz), see Tables 2 and 1; positive HRESIMS *m/z* 373.1963 ([*M* + Na]⁺, calcd for C₂₀H₃₀O₅Na, 373.1985). Crystal data for 2, size 0.19 \times 0.15 \times 0.12 mm³, orthorhombic, space group *P*₂1, *a* = 6.6713 (2) Å, *b* = 19.5694 (7) Å, *c* = 7.1999 (2) Å, *V* = 939.04 (5) Å³, *T* = 293.0 K, *Z* = 2, *D* = 1.303 Mg m^{−3}, λ (CuK α) = 1.54178 Å, *F*(000) = 400, reflections collected/unique 11 475/3259 [*R*(int) = 0.0428], *h* (−8/8), *k* (−23/23), *l* (−8/8), final *R* indices *R*₁ = 0.0492 and *wR*₂ = 0.1288 (*I* > 2 σ (*I*)), *R*₁ = 0.0503 and *wR*₂ = 0.1304 (all data), GOF = 1.040, largest diff. peak/hole, 0.459/−0.227 e Å^{−3}. Absolute structure parameter 0.02(9), CCDC: 2127070.†

Noueinsiancin C (3). White power; $[\alpha]_D^{25}$ −80.3 (*c* 0.065, CH₃OH); IR (KBr) ν_{\max} 3412, 2960, 2916, 2870, 1732, 1690, 1602, 1466, 1447, 1252, 1196, 1088, 1013, 996, 789 cm^{−1}; ¹H- and ¹³C-



NMR data (500 MHz/125 MHz), see Tables 2 and 1; positive HRESIMS m/z 373.1960 ($[M + Na]^+$, calcd for $C_{20}H_{30}O_5Na$, 373.1985).

Noeinsiancin D (4). Colorless needle crystals; $[\alpha]_D^{25} -108.3$ (c 0.06, CH_3OH); IR (KBr) ν_{max} 3199, 2945, 2872, 1738, 1708, 1466, 1449, 1245, 1172, 1047, 976, 795, cm^{-1} ; 1H - and ^{13}C -NMR data (500 MHz/125 MHz), see Tables 2 and 1; positive HRESIMS m/z 357.2023 ($[M + Na]^+$, calcd for $C_{20}H_{30}O_4Na$, 357.2036). Crystal data for **4**: size $0.200 \times 0.150 \times 0.130$ mm³, orthorhombic, space group $P2_1$, $a = 11.7128$ (3) Å, $b = 7.9902$ (2) Å, $c = 11.8269$ (3) Å, $V = 1001.54$ (4) Å³, $T = 293$ K, $Z = 2$, $D = 1.215$ Mg m⁻³, $\lambda(CuK\alpha) = 1.541789$ Å, $F(000) = 400$, reflections collected/unique 123 977/3447 [$R(int) = 0.0331$], h (−13/13), k (−8/9), l (−13/14), final R indices $R_1 = 0.0302$ and $wR_2 = 0.0800$ ($I > 2\sigma(I)$), $R_1 = 0.0310$ and $wR_2 = 0.0911$ (all data), GOF = 1.047, largest diff. peak/hole, 0.124/−0.094 e Å⁻³. Absolute structure parameter 0.09(6), CCDC: 2090338.†

Noeinsiancin E (5). Colorless needle crystals; $[\alpha]_D^{25} -140.2$ (c 0.10, CH_3OH); IR (KBr) ν_{max} 3370, 2964, 2869, 1714, 1448, 1366, 1288, 1169, 1018, 976, 755, cm^{-1} ; 1H - and ^{13}C -NMR data (500 MHz/125 MHz), see Tables 2 and 1; positive HRESIMS m/z 357.2023 ($[M + Na]^+$, calcd for $C_{20}H_{30}O_4Na$, 357.2036). Crystal data for **5**: size $0.200 \times 0.160 \times 0.130$ mm³, orthorhombic, space group $P1$, $a = 6.4700$ (4) Å, $b = 7.6635$ (5) Å, $c = 9.8461$ (6) Å, $V = 447.19$ (5) Å³, $T = 293$ K, $Z = 1$, $D = 1.242$ Mg m⁻³, $\lambda(CuK\alpha) = 1.541789$ Å, $F(000) = 182$, reflections collected/unique 13 755/2977 [$R(int) = 0.0359$], h (−7/7), k (−9/9), l (−11/11), final R indices $R_1 = 0.0304$ and $wR_2 = 0.0799$ ($I > 2\sigma(I)$), $R_1 = 0.0305$ and $wR_2 = 0.0800$ (all data), GOF = 1.069, largest diff. peak/hole, 0.138/−0.097 e Å⁻³. Absolute structure parameter 0.07(4), CCDC: 2090337.†

Noeinsiancin F (6). Colorless needle crystals; $[\alpha]_D^{25} -116.7$ (c 0.05, CH_3OH); IR (KBr) ν_{max} 3426, 2931, 1723, 1467, 1447, 1373, 1270, 1168, 1065, 977, 787 cm^{-1} ; 1H - and ^{13}C -NMR data (500 MHz/125 MHz), see Tables 2 and 1; positive HRESIMS m/z 355.1861 ($[M + Na]^+$, calcd for $C_{20}H_{28}O_4Na$, 355.1880). Crystal data for **6**: size $0.15 \times 0.07 \times 0.05$ mm³, orthorhombic, space group $P12_1$, $a = 6.4502$ (3) Å, $b = 14.2302$ (6) Å, $c = 9.9729$ (4) Å, $V = 905.16$ (7) Å³, $T = 293$ K, $Z = 2$, $D = 1.220$ Mg m⁻³, $\lambda(CuK\alpha) = 1.34139$ Å, $F(000) = 360$, reflections collected/unique 11 155/3409 [$R(int) = 0.0564$], h (−7/7), k (−17/17), l (−12/12), final R indices $R_1 = 0.0407$ and $wR_2 = 0.1083$ ($I > 2\sigma(I)$), $R_1 = 0.0431$ and $wR_2 = 0.1090$ (all data), GOF = 1.080, largest diff. peak/hole, 0.138/−0.097 e Å⁻³. Absolute structure parameter 0.01(11), CCDC: 2156441.†

Noeinsiancin G (7). White power; $[\alpha]_D^{25} -139.3$ (c 0.15, CH_3OH); IR (KBr) ν_{max} 3245, 2934, 2874, 1770, 1470, 1447, 1372, 1271, 1172, 1065, 976, 789 cm^{-1} ; 1H - and ^{13}C -NMR data (500 MHz/125 MHz), see Tables 3 and 1; positive HRESIMS 355.1860 ($[M + Na]^+$, calcd for $C_{20}H_{28}O_4Na$, 355.1880).

Noeinsiancin H (8). Colorless needle crystals; $[\alpha]_D^{25} -85.8$ (c 0.06, CH_3OH); IR (KBr) ν_{max} 3253, 2926, 2857, 1700, 1596, 1447, 1394, 1297, 1167, 1079, 976, 758 cm^{-1} ; 1H - and ^{13}C -NMR data (500 MHz/125 MHz), see Tables 3 and 1; positive HRESIMS m/z 357.2023 ($[M + Na]^+$, calcd for $C_{20}H_{30}O_4Na$, 357.2036). Crystal data for **8**: size $0.05 \times 0.105 \times 0.01$ mm³, orthorhombic, space group $P2_12_12_1$, $a = 7.4299$ (10) Å, $b = 10.3592$ (2) Å, $c = 23.4977$

(3) Å, $V = 1808.57$ (5) Å³, $T = 173$ K, $Z = 4$, $D = 1.294$ Mg m⁻³, $\lambda(CuK\alpha) = 1.34139$ Å, $F(000) = 768$, reflections collected/unique 129 313 420 [$R(int) = 0.0399$], h (−8/9), k (−12/12), l (−24/28), final R indices $R_1 = 0.0576$ and $wR_2 = 0.0799$ ($I > 2\sigma(I)$), $R_1 = 0.0305$ and $wR_2 = 0.0800$ (all data), GOF = 1.069, largest diff. peak/hole, 0.138/−0.097 e Å⁻³. Absolute structure parameter 0.07(4), CCDC: 2090340.†

Noeinsiancin I (9). Colorless needle crystals; $[\alpha]_D^{25} -91.3$ (c 0.05, CH_3OH); IR (KBr) ν_{max} 3421, 2941, 1695, 1467, 1444, 1370, 1284, 1160, 1066, 990, 793 cm^{-1} ; 1H - and ^{13}C -NMR data (500 MHz/125 MHz), see Tables 3 and 1; positive HRESIMS m/z 375.2123 ($[M + Na]^+$, calcd for $C_{20}H_{32}O_5Na$, 375.2142). Crystal data for **9**: size $0.20 \times 0.14 \times 0.10$ mm³, orthorhombic, space group $P2_1$, $a = 8.4122$ (2) Å, $b = 19.7326$ (6) Å, $c = 11.0321$ (3) Å, $V = 1829.87$ (9) Å³, $T = 293$ K, $Z = 4$, $D = 1.279$ Mg m⁻³, $\lambda(CuK\alpha) = 1.54178$ Å, $F(000) = 768$, reflections collected/unique 21 478/6401 [$R(int) = 0.0438$], h (−10/10), k (−23/23), l (−12/13), final R indices $R_1 = 0.0322$ and $wR_2 = 0.0841$ ($I > 2\sigma(I)$), $R_1 = 0.0327$ and $wR_2 = 0.0846$ (all data), GOF = 1.052, largest diff. peak/hole, 0.151/−0.138 e Å⁻³. Absolute structure parameter −0.02(5), CCDC: 2127071.†

Noeinsiancin J (10). White power; $[\alpha]_D^{25} -87.0$ (c 0.10, CH_3OH); IR (KBr) ν_{max} 3366, 2937, 2898, 1696, 1470, 1408, 1384, 1325, 1264, 1180, 1051, 996, 798 cm^{-1} ; 1H - and ^{13}C -NMR data (500 MHz/125 MHz), see Tables 3 and 1; positive HRESIMS m/z 375.2121 ($[M + Na]^+$, calcd for $C_{20}H_{32}O_5Na$, 375.2142).

Noeinsiancin K (11). White power; $[\alpha]_D^{25} -96.0$ (c 0.05, CH_3OH); IR (KBr) ν_{max} 3483, 2950, 2869, 1721, 1683, 1469, 1402, 1368, 1230, 1160, 1038, 977, 803 cm^{-1} ; 1H - and ^{13}C -NMR data (500 MHz/125 MHz), see Tables 3 and 1; positive HRESIMS m/z 373.1966 ($[M + Na]^+$, calcd for $C_{20}H_{30}O_5Na$, 373.1985).

2.5 Cell culture and cytotoxicity assay

The RAW 264.7 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS, 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin at 37 °C, 5% CO₂. All the test compounds were dissolved in dimethyl sulfoxide (DMSO) and freshly prepared each time before use. Then, RAW 264.7 cells were seed at 96-well plates at a density of 5×10^4 cells per well cultured overnight. The cells were treated with the test compounds (2.5, 5.0 and 10.0 µM) and LPS (1 µg mL⁻¹), respectively. After 24 h incubation, 10 µL CCK-8 solution was added to each well and incubated at 37 °C for 30 min. Finally, the optical OD-value was measured at 450 nm through a microplate reader, respectively (BioTek Instruments, Inc.).

2.6 Nitric oxide production inhibitory activity assay

The nitric oxide (NO) level was determined through measuring the concentration of nitrite in culture medium using Griess assay. RAW 264.7 cells were seeded in 96-well plates (5×10^4 cells per well) for 24 h, then, the cells were treated with or without the test compounds (2.5, 5.0 and 10.0 µM) for 2 h prior to LPS (1 µg mL⁻¹). After incubation for 24 h, 100 µL supernatant mixed with 100 µL Griess reagent and incubated for 20 min



at 37 °C in the dark. Finally, the absorbance was measured at 540 nm through a microplate reader, respectively (BioTek Instruments, Inc.).

2.7 The detection of cytokines production

RAW 264.7 cells were seeded in 12-well plates (2×10^5 cells per well) for 24 h and the cells were also treated with or without the test compounds at the concentration of 2.5, 5.0 and 10.0 μM for 2 h before LPS ($1 \mu\text{g mL}^{-1}$) stimulation. After incubation for 24 h, the culture media were collected. Then, the levels of TNF- α and IL-6 were measured using the ELISA kits (Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer's instruction.

2.8 Statistical analysis

Prism 8 (GraphPad, San Diego, CA, USA) were used for statistical analysis. Dates of three separate studies were analysed by the one-way ANOVA test and the Tukey *post hoc* test. Meanwhile, all data expressed as mean \pm standard deviation (SD), which collected from three independent experiments. The significant difference was determined with $P < 0.05$.

3. Results and discussion

3.1 Structural identification

Compound **1** was isolated as colorless needle crystals from MeOH/H₂O. Its molecular formula, C₂₀H₃₀O₅, was deduced from the positive HRESIMS ion peak at m/z 373.1962 [$M + Na$]⁺, calcd for C₂₀H₃₀O₅Na, 373.1985), corresponding to six indices of hydrogen deficiency. Analysis of the ¹H NMR spectra of **1** (Table 2) showed characteristic signals assignable to three methyl singlets at δ_{H} 1.34 (s, H₃-17), 1.19 (s, H₃-19) and 0.99 (s, H₃-20),

as well as three oxymethine protons at δ_{H} 4.09 (1H, H-11), 4.14 (1H, H-12) and 2.82 (1H, H-15). The ¹³C NMR and DEPT spectra (Table 1) indicated the presence of 20 carbon signals, including three methyls (δ_{C} 18.8, 21.0, 29.6); six methylenes (δ_{C} 20.2, 22.2, 35.1, 37.3, 39.1 and 42.6); six methines (including three oxygenated at δ_{C} 79.8, 82.3 and 82.5, three sp³ carbon signals at δ_{C} 50.7, 52.3 and 57.9) and five quaternary carbons [δ_{C} 37.8, 44.5, 45.6, 85.4 and 181.6 (a carboxyl group)]. Additionally, according to the ¹H and ¹³C NMR spectra of **1**, six indices of hydrogen deficiency were attributed to one carbonyl group (δ_{C} 181.6) and one epoxy group [δ_{H} 4.09, H-11 and δ_{C} 82.5, C-11; 85.4, C-16]. The remaining four ones exhibited the existence of a tetracyclic system in **1**.⁷ Consequently, the four-ring was inferred to be a 6/6/6/5 fused rings A, B, C and D, a characteristic *ent*-kaurane diterpenoid skeleton,^{8–10} based on the 2D NMR experiments (Fig. 2): the ¹H–¹H COSY correlations of H₂-1/H₂-2/H₂-3, H-5/H₂-6/H₂-7 and H-9/H-11/H-12/H-13/H₂-14; the HMBC correlations from H₃-19 to C-3, C-4, C-5 and C-18; from H₃-20 to C-1, C-5, C-9 and C-10; from H₂-7 to C-6 and C-8; from H-9 to C-5, C-8, C-10, C-11 and C-12; from H₂-14 to C-8, C-9, C-13, C-15 and C-16; and from H₃-17 to C-13, C-15 and C-16. In addition, the occurrence of a 11,16-epoxy motif in **1** was determined by the HMBC correlations of H-11 with C-16. Thus, the planar structure of **1** was determined.

The relative configuration of **1** was determined through the NOESY spectrum (Fig. 3). The NOESY correlations of H₃-19/H-5/H-9 indicated they were assigned the β -orientation. Meanwhile, H₃-20, H-12 and H-11, H-13, H-15 were shown to be in a α -orientation according to the NOESY interactions from H₃-20 to H-12 and H₃-20 to H-11, H-13 and H-15. In order to determine its structure and absolute configuration, an appropriate crystal of **1** was obtained from a solvent system of MeOH/H₂O after many single crystal cultures.

Table 1 ¹³C NMR spectroscopic data for compounds **1**–**11**^a (δ in ppm)

No.	1	2	3	4	5	6	7	8	9	10	11
1	42.6	35.7	41.2	41.1	49.7	49.9	35.0	33.9	42.0	40.3	34.4
2	20.2	27.1	18.8	20.1	64.7	64.8	27.2	25.9	20.3	18.9	27.0
3	39.1	71.5	37.7	39.0	47.7	47.7	72.0	70.3	39.3	37.8	71.5
4	44.5	48.3	43.0	44.7	45.8	45.9	45.0	47.7	44.7	43.3	48.6
5	57.9	50.3	56.7	57.3	56.9	56.9	49.6	48.3	57.7	56.4	49.3
6	22.2	21.9	20.9	21.4	21.4	21.2	21.1	21.0	23.1	21.4	21.1
7	35.1	35.9	34.4	34.5	35.4	34.9	34.9	38.7	36.0	34.3	35.2
8	45.6	46.6	45.3	53.5	53.9	53.7	54.0	45.6	49.3	45.9	54.5
9	52.3	51.6	51.0	52.3	53.1	53.1	53.2	45.4	47.6	46.0	53.1
10	37.8	37.6	36.5	40.8	42.4	42.7	41.4	38.8	40.4	39.0	41.0
11	82.5	77.8	76.3	29.0	19.4	19.6	19.5	17.5	19.7	18.5	19.5
12	79.8	39.7	38.2	71.9	25.6	33.2	33.4	33.1	27.5	26.2	25.8
13	50.7	43.3	38.3	49.4	36.3	39.4	39.5	40.1	45.0	39.5	33.6
14	37.3	38.9	37.5	37.5	38.2	37.7	37.7	36.1	41.6	40.6	38.0
15	82.3	82.9	77.7	226.2	227.0	212.3	212.8	81.8	90.4	79.9	224.0
16	85.4	85.6	86.8	42.9	49.0	151.2	151.4	157.6	84.1	77.1	57.3
17	21.0	20.7	61.8	11.7	10.3	115.4	115.1	103.7	64.6	69.1	59.9
18	181.6	181.3	180.2	181.3	180.7	181.2	183.0	180.2	181.7	180.3	181.0
19	29.6	25.1	28.2	29.4	29.3	29.5	25.3	23.6	29.5	28.1	24.9
20	18.8	18.2	17.0	16.3	17.2	17.3	16.2	14.8	16.3	14.7	15.8

^a NMR data were measured at 125 MHz in CD₃OD for **1**–**11**.



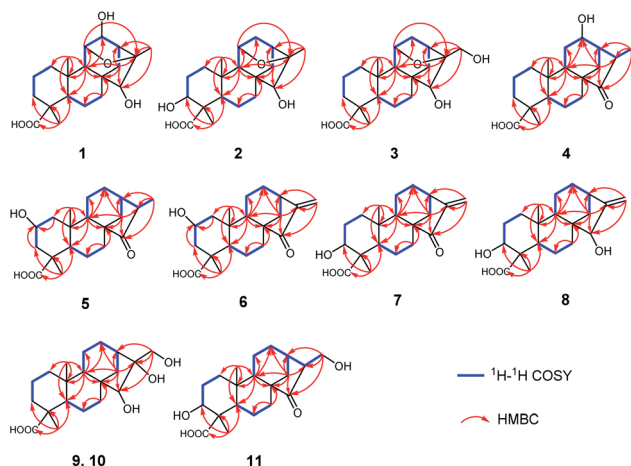
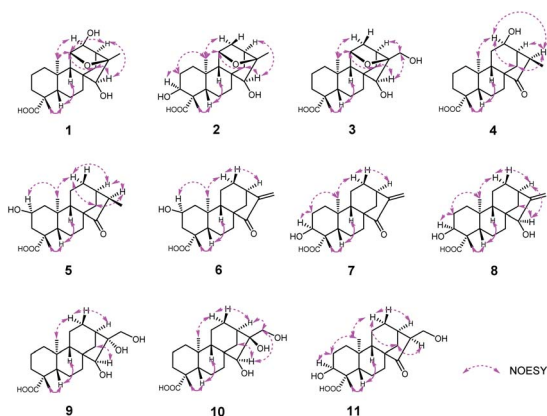
Fig. 2 The ^1H – ^1H COSY and HMBC correlations of 1–11.

Fig. 3 The key NOESY correlations of 1–11.

Therefore, the X-ray crystallographic analysis with Cu K α radiation unambiguously confirmed the absolute configuration of **1** was 4*R*, 5*S*, 8*R*, 9*S*, 10*R*, 11*R*, 12*S*, 13*S*, 15*S*, 16*R* (Fig. 4). Finally, the structure of **1** was ascertained completely and named noueinsiancin A.

Compound **2**, obtained as colorless needle crystals, possessed the same molecular formula of $\text{C}_{20}\text{H}_{30}\text{O}_5$ as **1** based

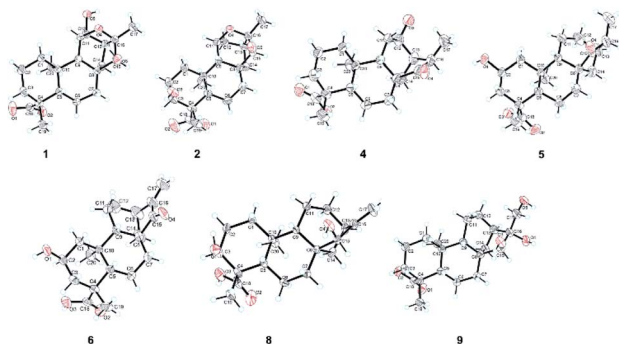


Fig. 4 The X-ray crystallographic structures of 1, 2, 4–6, 8 and 9.

on the ion peak at m/z 373.1963 ($[\text{M} + \text{Na}]^+$, calcd for $\text{C}_{20}\text{H}_{30}\text{O}_5\text{Na}$, 373.1985) in the positive HRESIMS spectrum. Overall analysis of the 1D NMR (Tables 1 and 2) and 2D NMR (^1H – ^1H COSY and HMBC) spectroscopic spectrum indicated that the gross structure of **2** was nearly close to that of **1**. In fact, the only difference between them was the presence of a hydroxy group at C-3 (δ_{C} 71.5) position in **2** instead of the hydroxy group at C-12 (δ_{C} 79.8) position in **1**. This was verified by the ^1H – ^1H COSY correlations of H_2 -1/ H_2 -2/ H -3 (δ_{H} 3.99) and H -9/ H -11/ H_2 -12 (δ_{H} 2.00, 1.82)/ H -13/ H_2 -14, together with the HMBC correlations from H_3 -19 to C-3 (δ_{C} 71.5), C-4 (δ_{C} 48.3) and C-5 (δ_{C} 50.3) (Fig. 2). The analysis of NOESY data revealed that the molecular conformation of **2** was identical to that of **1** (Fig. 3). Similarly, to further determine the structure and absolute configuration of **2**, a suitable crystal of **2** for the single-crystal X-ray diffraction experiment (Cu K α) was obtained. Finally, its absolute configuration was confirmed as 3*S*, 4*S*, 5*S*, 8*R*, 9*S*, 10*R*, 11*S*, 13*S*, 15*S*, 16*R* (Fig. 4). Hence, the structure of **2** was defined and named noueinsiancin B.

Compound **3** was isolated as a white amorphous powder and showed a molecular formula of $\text{C}_{20}\text{H}_{30}\text{O}_5$ according to the positive HRESIMS data: 373.1960 ($[\text{M} + \text{Na}]^+$, calcd for $\text{C}_{20}\text{H}_{30}\text{O}_5\text{Na}$, 373.1985). Detailed comparison of the ^1H and ^{13}C NMR spectra of **3** and **1** (Tables 1 and 2) revealed that the structure of **3** was similar to that of **1**. The main difference was that the absence of a hydroxy group at C-12 position in **3** and the replacement of the methyl group in **1** at C-16 position by a hydroxy-methylene group in **3**. This conclusion was supported by the hydroxyl-methylene signal at δ_{C} 61.8 (C-17) and the HMBC correlations from H_2 -17 (δ_{H} 3.59) to C-13 (δ_{C} 38.3), C-16 (δ_{C} 86.8) and C-15 (δ_{C} 77.7) (Fig. 2). Additionally, detailed analysis of the NOESY data of **3** indicated its relative configuration was also the same as **1**. Aim to elucidated its absolute configuration, ECD calculations, a method regarded as a powerful and effective method to confirm the absolute configuration of the natural products,^{11,12} was applied. Subsequently, its absolute configuration was established as 4*R*, 5*S*, 8*R*, 9*S*, 10*R*, 11*S*, 13*S*, 15*S*, 16*S* by comparing the calculated and experimental ECD spectrum (Fig. 5). Consequently, the structure of **3** was obtained and named noueinsiancin C.

Compound **4** was initially purified as colorless needle crystals and assigned a molecular formula of $\text{C}_{20}\text{H}_{30}\text{O}_4$ from the $[\text{M} + \text{Na}]^+$ ion peak at m/z 357.2023 (calcd for $\text{C}_{20}\text{H}_{30}\text{O}_4\text{Na}$, 357.2036) in positive HRESIMS. The 1D NMR data of **4** (Tables 1 and 2) were closely similar to those of **1**, except for the absence of the 11,16-epoxy group in **4** and the substitution of C-15 position (the hydroxy group for the former, and the carbonyl group for the latter). These above deductions were revealed by the ^1H – ^1H COSY correlations of H -9/ H_2 -11/ H -12/ H -13/ H_2 -14 and H -13/ H -16/ H_3 -17, together with the HMBC correlations from H_3 -17 (δ_{H} 1.25, d, $J = 7.2$ Hz) to C-13 (δ_{C} 49.4), C-16 (δ_{C} 42.9) and C-15 (δ_{C} 226.2) (Fig. 2). In the NOESY spectrum (Fig. 3), the correlations of H_3 -19/ H -5/ H -9 and H_3 -20/ H -12/ H -16/ H_2 -14 demonstrated the β -configuration and α -configuration of these protons, respectively. Finally, by means of the single-crystal X-ray diffraction analysis with Cu K α radiation (Fig. 4), the absolute configuration of **4** was assigned as 4*R*, 5*S*, 8*R*, 9*S*, 10*S*, 12*R*,



Table 2 ^1H NMR spectroscopic data for compounds 1–6^a (δ in ppm, J in Hz)

No.	1	2	3	4	5	6
1	1.88, 1.19	1.58	1.85, 1.18	1.86, 0.85	2.17, 0.67, t, (12.0)	2.19, 0.70, t, (12.0)
2	1.85, 1.43, m	2.13, 1.57, m	1.86, 1.42, m	1.91, 1.42, m	4.08, m	4.09, m
3	2.16, 1.05, m	3.99, overlap	2.16, 1.04, m	2.13, 1.04, m	2.41, 0.96, t, (12.2)	2.41, 0.97, t, (12.3)
5	1.05, m	1.47, m	1.06, m	1.17, m	1.11, m	1.11, m
6	1.84, 1.70, m	1.76, m	1.85, 1.75, m	1.90, 1.78, m	1.93, 1.81, m	1.95, m
7	1.54, 1.25, m	1.78, 1.21, m	1.56, 1.22, m	1.74, 1.39, m	1.67, 1.36, m	1.82, 1.34, m
9	1.72	1.68	1.70	1.16	1.06	1.19
11	4.09, overlap	4.28, overlap	4.33, overlap	1.93, m	1.71, 1.23, m	1.77, 1.47, m
12	4.14, overlap	2.00, d, (11.4), 1.82	2.00, d, (11.2), 1.80	4.09, m	1.69, m	1.89, 1.69, m
13	2.14	2.19	2.45	2.41, m	2.44, m	3.07, m
14	1.95, d, (12.7), 1.19	1.95, d, (12.3), 1.14	1.97, d, (12.3), 1.11	2.33, d, (12.5), 1.48	2.45, overlap, 1.43	2.43, overlap, 1.40
15	2.82, s	2.87, s	3.06, s			
16				2.59, m	2.30, m	
17	1.34, s	1.27, s	3.59 dd, (11.5, 5.8)	1.25, d, (7.2)	1.09, d, (7.2)	5.89, 5.32, s
19	1.19, s	1.24, s	1.19, s	1.21, s	1.27, s	1.27, s
20	0.99, s	1.01, s	1.02, s	0.98, s	1.04, s	1.06, s

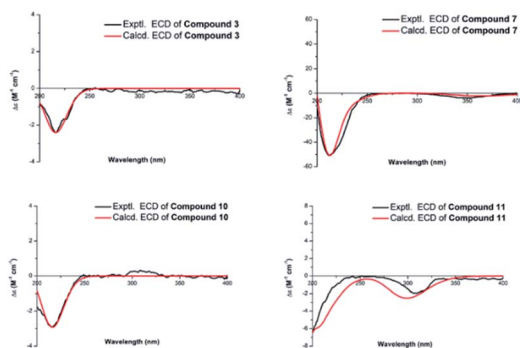
^a NMR data were measured at 500 MHz in CD_3OD for 1–6.

Fig. 5 Experimental and calculated ECD spectra of compounds 3, 7, 10 and 11.

13R, 16R. Consequently, the structure of 4 was determined named noueinsiancin D.

Compound 5, isolated as colorless needle crystals, provided the same molecular formula as that of 4 by its positive HRESIMS. Comparison of the 1D NMR spectra (^1H , ^{13}C and DEPT) of 5 (Tables 1 and 2) with those of 4, followed by the detailed analysis the 2D NMR spectra (^1H – ^1H COSY and HMBC) established that the structure of 5 was close to that of 4. The main difference between 5 and 4 was in the positioning of a hydroxy group, which in 5 was at C-2 position and in 4 was at C-12 position. This ratiocination was corroborated by the ^1H – ^1H COSY correlations of H_2 -1 (δ_{H} 2.17, 0.67)/ H_2 -2 (δ_{H} 4.08)/ H_2 -3 (δ_{H} 2.41, 0.96), as well as the HMBC cross-peaks from H_3 -19 (δ_{H} 1.27, s) to C-3 (δ_{C} 47.7) methylene, from H_3 -20 (δ_{H} 1.04, s) to C-1 (δ_{C} 49.7) methylene and from H_2 -3 (δ_{H} 2.41, 0.96) and H_2 -1 (δ_{H} 2.17, 0.67) both to C-2 (δ_{C} 64.7) (Fig. 2). Moreover, the crucial NOESY interactions of H_2 /H₃-20 and H₃-20/H-12 α /H₂-14/H-16 (Fig. 3) confirmed that these protons were both α -oriented and the configuration of 2-OH was β -oriented. Its absolute configuration was 2S, 4R, 5S, 8R, 9S, 10S, 13R, 16R as consolidated by the single crystal X-ray diffraction with Cu K α radiation

(Fig. 4) Finally, the structure of 5 was defined and named noueinsiancin E.

Compound 6 was isolated as colorless needle crystals. Its molecular formula ($\text{C}_{20}\text{H}_{28}\text{O}_4$) was deduced by analysing the positive HRESIMS (m/z 355.1861 [$\text{M} + \text{Na}$] $^+$, calcd for $\text{C}_{20}\text{H}_{28}\text{O}_4\text{Na}$, 355.1880). By detailed comparison of the ^1H and ^{13}C NMR spectra (Tables 1 and 2) between 6 and 5 indicated that the closely related structures of them, except for the exocyclic $\Delta^{16(17)}$ double bond in 6 instead of the methine (C-16, δ_{C} 49.0) and the methyl (CH_3 -17, δ_{C} 10.3) in 5. This conclusion was evidenced by the HMBC correlations from H_2 -17 (δ_{H} 5.89, 5.32, 2H) to C-13 (δ_{C} 39.4), C-16 (δ_{C} 151.2) and C-15 (δ_{C} 212.3). In the NOESY data (Fig. 3), the correlations of H_3 -19/H-5/H-9 verified the β -orientation of Me-19, H-5 and H-9, while the NOESY correlations of H_2 /H₃-20/H-12 α /H-13 assigned H-2, Me-20 and H-13 as α -orientated. Then, the absolute configuration of 6 was confirmed as 2S, 4R, 5S, 8R, 9S, 10S, 13R on the basis of the single crystal X-ray diffraction experiment with Cu K α radiation (Fig. 4). Hence, the structure of 6 was obtained and named noueinsiancin F.

Compound 7 was assigned an equal molecular formula ($\text{C}_{20}\text{H}_{28}\text{O}_4$) to that of 6 from the ion peak at m/z 355.1860 ([$\text{M} + \text{Na}$] $^+$, calcd for $\text{C}_{20}\text{H}_{28}\text{O}_4\text{Na}$, 355.1880) in the positive HRESIMS spectrum and ^{13}C NMR data. Detailed interpretation of its 1D NMR (Tables 1 and 3) and 2D NMR data indicated that the structure of 6 was highly resembled that of 5 with different positioning of a hydroxy group (the hydroxy group at the C-3 (δ_{C} 72.0) position for 7, and the hydroxy group at the C-2 (δ_{C} 64.8) position in 6). This deduction was determined by the ^1H – ^1H COSY correlations of H_2 -1 (δ_{H} 1.54, 1.24)/ H_2 -2 (δ_{H} 2.24, 1.53)/H-3 (δ_{H} 4.00) and the HMBC correlations from H_3 -19 (δ_{H} 1.24, 3H) to C-3 (δ_{C} 72.0), C-4 (δ_{C} 45.0) and C-5 (δ_{C} 49.6). Additionally, the NOESY spectrum (Fig. 3) suggested that 7 had the same relative configuration as 6, where the H-3 and H₃-20 assigned the α -orientation by the NOESY correlations from H-3 to H_2 -2 and H₃-20. Its absolute configuration was determined to be 3S, 4S, 5S, 8R, 9S, 10S, 13R based on the comparison of the calculated and



Table 3 ^1H NMR spectroscopic data for compounds 7–11^a (δ in ppm, J in Hz)

No.	7	8	9	10	11
1	1.54, 1.24	1.63, 1.32	1.90, 0.89	1.92, 0.89	1.55, 1.23
2	2.24, 1.53, m	2.19, 1.55, m	1.91, 1.40, m	1.91, 1.40, m	2.18, 1.54, m
3	4.00	3.98	2.12, 1.02, m	2.12, 1.01, m	3.97, overlap
5	1.54, m	1.48, m	1.05, m	1.04, m	1.57, m
6	1.92, 1.80, m	1.86, 1.73, m	1.83, m	1.82, 1.60, m	1.86, 1.76, m
7	1.82, 1.31, m	1.65, 1.29, m	1.91, 1.45, m	1.94, 0.88, m	1.72, 1.34, m
9	1.22	1.44	1.40	1.40	1.14
11	1.72, 1.43, m	1.63, 1.49, m	1.61, 1.53, m	1.69, 1.47, m	1.69, 1.21, m
12	1.89, 1.67, m	1.63, 1.45, m	1.63, 1.47, m	1.76, 1.40, m	1.75, m
13	3.06, m	2.59, m	1.92, m	1.96, m	2.65, m
14	2.46, d, (12.1), 1.38	2.00, d, (11.9), 1.01	1.58, 1.42	1.60, 1.33	2.50, d, (12.2), 1.42
15		3.68	3.42, s	3.06, s	
16					2.50
17	5.88, 5.30, s	5.04, s, 4.91, overlap	3.75 dd, (11.9, 10.2)	3.42, d, (11.2), 3.27 d, (11.3)	3.94, dd, (11.3, 6.6), 3.59, dd, (9.2, 2.1)
19	1.24, s	1.23, s	1.18, s	1.18, s	1.24, s
20	1.08, s	0.99, s	0.99, s	0.97, s	1.05, s

^a NMR data were measured at 500 MHz in CD_3OD for 7–11.

experimental ECD spectra (Fig. 5). Thus, the structure of compound 7 was established and named noueinsiancin G.

The molecular formula of 8 was $\text{C}_{20}\text{H}_{30}\text{O}_4$ as deduced from the ion peak at m/z 357.2023 ($[\text{M} + \text{Na}]^+$, calcd for $\text{C}_{20}\text{H}_{30}\text{O}_4\text{Na}$, 357.2036) in the positive HRESIMS spectrum. The ^1H and ^{13}C NMR spectroscopic data (Tables 1 and 3) of 8 were found to be very identical with those of 7, indicating that the structure of 8 was nearly similar to that of 7. Unlike compound 7, the signal ascribed to the carbonyl group at C-15 position were not observed for 8. Instead, a hydroxy group (δ_{H} 3.68; δ_{C} 81.8) was present at C-15 position in 8. This was determined by the HMBC correlations from H_2 -17 (δ_{H} 5.04, 4.91) to C-15 (δ_{C} 81.8), C-13 (δ_{C} 40.1) and C-16 (δ_{C} 157.6) (Fig. 2). Similarly, the NOESY data was also showed their same relative configuration, and the OH-15 was assigned as β -oriented verified by the NOESY cross-peaks from H_3 -20 to H-12 α , H-13 and H-15 (Fig. 3). The absolute configuration of 8 was determined as 3*S*, 4*S*, 5*S*, 8*R*, 9*S*, 10*S*, 13*R*, 15*R* by the single crystal X-ray diffraction experiment with Cu K α radiation (Fig. 4). Consequently, its structure was defined and named noueinsiancin H.

Compound 9 was obtained as colorless needle crystals, with a molecular formula of $\text{C}_{20}\text{H}_{32}\text{O}_5$ based on the quasi-molecular ion peak at m/z 375.2123 ($[\text{M} + \text{Na}]^+$, calcd for $\text{C}_{20}\text{H}_{32}\text{O}_5\text{Na}$, 375.2142) in its positive HRESIMS data. A detailed analysis revealed the ^1H and ^{13}C NMR spectra (Tables 1 and 3) of 9 were similar to those of 8, except for the absence of a hydroxyl group at C-3 position and a 16, 17-diol functional group¹³ instead of an exocyclic double bond at C-16 and C-17 position in 9. This deduction was subsequently verified the ^1H - ^1H COSY correlations from H_2 -1/ H_2 -2/ H_2 -3, together with the HMBC correlations from H_2 -17 (δ_{H} 3.75) to C-13 (δ_{C} 45.0) C-15 (δ_{C} 90.4) and C-16 (δ_{C} 84.1) (Fig. 2). In the NOESY spectrum (Fig. 3), the correlations of H_3 -19/H-5/H-9 and H_3 -20/H-12 α /H-13 verified the β - and α -orientation of these protons. However, the relative configuration of the remaining chiral carbons could not be determined due to the lack of NOESY correlations. Then, a suitable crystal of

9 was obtained followed by the single-crystal X-ray diffraction experiment using the Cu K α radiation (Fig. 4). Finally, its absolute configuration was confirmed as 4*R*, 5*S*, 8*R*, 9*S*, 10*S*, 13*R*, 15*S*, 16*R*. Consequently, the structure of 9 was defined and named as noueinsiancin I.

The molecular formula of 10 ($\text{C}_{20}\text{H}_{32}\text{O}_5$) was possessed to be identical to that of 9 on the basis of the (+)-HRESIMS ion peak at m/z 375.2121 ($[\text{M} + \text{Na}]^+$, calcd for $\text{C}_{20}\text{H}_{32}\text{O}_5\text{Na}$, 375.2142). The planar structure of 10 was conjectured to be the same as 9 by the detailed analysis its NMR data (1D and 2D) (Fig. 2). However, further upon comparison of their ^{13}C NMR data, the main difference was the change in the chemical shifts of C-15, C-16, and C-17 from δ_{C} 90.4, 84.1 and 64.6 in 9 to 79.9, 77.1 and 69.1 in 10. The shieldings of C-15 ($\Delta\delta$ -10.5) and C-16 ($\Delta\delta$ -7.0) in 10 were clearly implied the configurational change of C-16. This deduction was determined by the NOESY correlations of H-15/ H_2 -17/H-13 (Fig. 3), suggesting these protons were on the same face and confirmed as α -orientation. Therefore, compound 10 was deduced as the 16-epimer of compound 9. By comparing the calculated and experimental ECD spectra (Fig. 5), the absolute configuration of 10 was confirmed as 4*R*, 5*S*, 8*R*, 9*S*, 10*S*, 13*R*, 15*S*, 16*S*. Hence, its structure was established and named noueinsiancin J.

Compound 11 was obtain as white amorphous powder and possessed a ion peak at m/z 373.1966 ($[\text{M} + \text{Na}]^+$, calcd for $\text{C}_{20}\text{H}_{30}\text{O}_5\text{Na}$, 373.1985) in the positive HRESIMS, corresponding to the molecular formula of $\text{C}_{20}\text{H}_{30}\text{O}_5$. The ^1H and ^{13}C NMR spectra (Tables 1 and 3) of 11 were closely similar to those of 7, with the difference being the presence of a hydroxy-methylene group (δ_{H} 3.94, 3.59, δ_{C} 59.9) instead of an exocyclic $\Delta^{16(17)}$ double bond at C-16 position in 11. This was corroborated by the clear ^1H - ^1H COSY correlations of H-13 (δ_{H} 2.65)/H-16 (δ_{H} 2.50)/ H_2 -17 (δ_{H} 3.94, 3.59), as well as the HMBC correlations from H_2 -17 (δ_{H} 3.94, 3.59) to C-15 (δ_{C} 224.0), C-16 (δ_{C} 57.3) and C-13 (δ_{C} 33.6) (Fig. 3). In addition, the NOESY spectrum suggested that the relative configuration of 11 was identical with 7.



H-16 was assigned to be α -orientation based on the NOESY correlations from H₃-20 to H-12 α , H₂-14 and H-16 (Fig. 3). The absolute configuration of **11** was assigned to be 3*S*, 4*S*, 5*S*, 8*R*, 9*S*, 10*S*, 13*R*, 16*S* based on by the high agreement between the calculated and experimental ECD curves (Fig. 5), accordingly, its structure was determined and named noueinsiancin K.

3.2 Nitric oxide production inhibitory activity

All the isolated compounds (**1**–**27**) were assayed for their inhibitory activities against NO production in LPS-stimulated RAW 264.7 cells. Compared with the LPS group, compounds **4**–**7** and **13**–**17** evidently decreased the NO production in LPS-induced RAW 264.7 cells (Fig. 6A). And, they possessed no significant cytotoxicity at their effective concentration for the inhibition of NO production (Fig. 6B). Notably, the new compounds **6** and **7** showed stronger inhibitory activities at 2.5, 5.0 and 10.0 μ M dose concentrations. Thus, we chose **6** and **7** as the material to further study on the anti-inflammatory activity.

The preliminary analysis of the structure–activity relationship on inhibitory activities against NO production in LPS-induced RAW 264.7 cells were discussed as follows: in all the tested compounds, we found that the five compounds (**6**, **7**, **15**–**17**) possessed the α -methylene- γ -carbonyl group at C-16 and C-15 positions, displaying much better inhibitory activities than others. Additionally, in compounds **4**, **5**, **13** and **14**, the methyl group instead of the α -methylene group at C-16 position would weaken the activity (**6**, **7**, **15**–**17** > **4**, **5**, **13**, **14**). Consequently, we inferred that the α -methylene- γ -carbonyl group was a functional structure for inhibiting NO production in LPS-induced RAW 264.7 cells.

3.3 **6** and **7** decreased the protein levels of TNF- α and IL-6 in LPS-induced RAW 264.7 cells

Due to the TNF- α and IL-6 were the important factors of inflammation.^{14–16} Therefore, the effects of **6** and **7** on the protein levels of IL-6 and TNF- α in LPS-induced RAW 264.7 cells were examined. After RAW 264.7 cells were induced by LPS (1 μ g

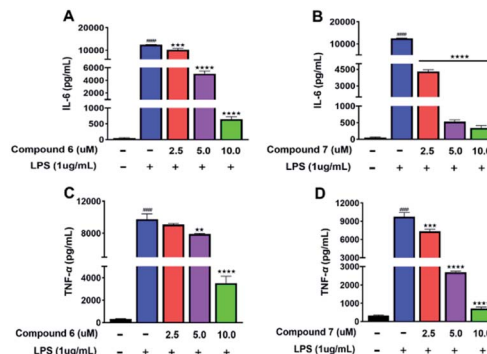


Fig. 7 (A and C) The effects of **6** on the protein expression levels of IL-6 and TNF- α in LPS-induced RAW 264.7 cells. (B and D) The effects of **7** on the protein expression levels of IL-6 and TNF- α in LPS-induced RAW 264.7 cells. All results were expressed as mean \pm S.D. ($n = 3$). #### $p < 0.0001$ vs. control group. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. the LPS group.

mL⁻¹) for 24 h with or without **6** and **7**, the culture media were collected. Then, the levels of TNF- α and IL-6 were measured using the ELISA kits. As shown in Fig. 7A–D. Compared with the normal group, treated LPS could evidently up-regulated the protein expressions of TNF- α and IL-6. Then, compounds **6** and **7** treatment led to an obvious downregulation in the above-mentioned protein expressions in a dose-dependent manner, comparing to the LPS-stimulated group.

4. Conclusions

In conclusion, noueinsiancins A–K (**1**–**11**), eleven new ent-kaurane diterpenoid acids, together with sixteen related known analogs (**12**–**27**) were isolated from *Nouelia insignis* Franch. The chemical structures including absolute configurations of the eleven new compounds were identified by the analyses of their NMR spectra, HRESIMS spectra, ECD calculation and single crystal X-ray diffraction. Moreover, the anti-inflammatory activity evaluation research revealed that compounds **4**–**7** and **13**–**17** significantly inhibited NO production at the concentration of 2.5 μ M, 5.0 μ M and 10.0 μ M in LPS-stimulated RAW 264.7 cells. At the same time, compounds **6** and **7** evidently reduced the protein expression levels of IL-6 and TNF- α in RAW 264.7 cells induced by LPS. Especially, the analysis of the structure–activity relationship indicated that the α -methylene- γ -carbonyl group was a functional structure for anti-inflammatory activity. Above all, our findings provide the idea that the administration of *Nouelia insignis* Franch may be beneficial for the treatment of inflammation disease.

Conflicts of interest

The authors declare no conflicts of interest in this paper.

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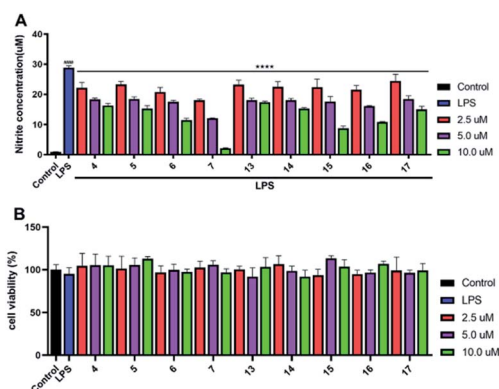


Fig. 6 NO inhibitory activities study *in vitro*, (A) the inhibitory effects of **4**–**7** and **13**–**17** on NO production in LPS-induced RAW 264.7 cells at 2.5, 5.0 and 10.0 μ M dose concentrations. (B) The cytotoxic effect of these compounds on RAW 264.7 cells by CCK8 assay. All results were expressed as mean \pm S.D. ($n = 3$). #### $p < 0.0001$ vs. control group. *** $p < 0.0001$ vs. the LPS group.



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