Vlasoulides A and B, a pair of neuroprotective C\(_{32}\) dimeric sesquiterpenes with a hexacyclic 5/7/5/5/(5)/7 carbon skeleton from the roots of Vladimiria souliei†

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Vlasoulides A and B (1 and 2), a pair of epimeric C\(_{32}\) sesquiterpene lactone dimers, featuring a 5/7/5/5/(5)/7 ring system were isolated from the roots of Vladimiria souliei. Their chemical structures were determined by comprehensive analysis of spectroscopic data, including HRESIMS and 1D and 2D NMR spectroscopic data. Their absolute configurations were established by Mosher’s method and ECD experiments. Furthermore, biological studies showed that compound 1 showed prominent neuroprotective effects against glutamate-induced neurotoxicity in PC-12 cells, with EC\(_{50}\) values of 13.54 ± 0.33 μM, while, the EC\(_{50}\) value of compound 2 is greater than 30 μM.

Introduction

The large genus Vladimiria belonging to the family Asteraceae comprises about 12 species. Most of them are widely distributed in East Asia, especially in the Sichuan Province, China.\(^1,2\) The roots are usually applied in traditional Chinese medicine to treat a number of diseases, showing antitumor, analgesic, anti-inflammatory, gastric ulcer resistant and antibacterial activities.\(^3\)–\(^6\) Furthermore, according to previous phytochemical studies, they were found to contain many sesquiterpenes, steroids, phenylpropanoids, flavonoids, and triterpenes.\(^7\)–\(^9\) Aiming to search for new and rare SLDs with unique skeletons and novel bioactivities from this genus Vladimiria, we investigated the chemical constituents of the roots of Vladimiria souliei. Over the past few years, some rare sesquiterpene lactone dimers (SLDs), with influence on NO production and the activation of NF-κB pathway,\(^10\)–\(^14\) were isolated from the title plant. At the same time, we also have separated some rare dimeric sesquiterpenes exhibiting potential neuroprotection activity from Vladimiria souliei in our previous study.\(^15,16\) Therefore, as a continuing investigation on this plant, two C\(_{32}\) sesquiterpene dimers, vlasoulides A and B (1 and 2), were isolated from the same plant. Herein, we describe the isolation, structural elucidation and neuroprotective activities of two new compounds (Fig. 1).

Fig. 1  Chemical structures of 1 and 2.
Plant material
The whole plant of *Vladimiria souliei* was collected at Dajin, Sichuan Province of China, in October, 2019, and authenticated by Prof. Bao-Kang Huang of Second Medical Military University. Currently a voucher specimen (No. 20191001) is deposited in School of Pharmacy, Second Military Medical University.

Extraction and isolation
The dried and chipped roots of *V. souliei* (50.0 kg) were extracted by maceration with 95% ethanol overnight at room temperature (3 × 60 L). After remove of solvent, the ethanol extract (5.60 kg) was partitioned between water and petroleum ether (PE)/ethyl acetate (EtOAc), successively, to give PE, EtOAc and water extracts. EtOAc extract (1.15 kg) was further purified by MCI column chromatography (MeOH/H2O, 30 : 70 to 100 : 0) to give 8 fractions (Fr. 1–8). Fraction 5 (52.5 g) was further isolated by ODS column chromatography (MeOH/H2O, 30 : 70 to 90 : 10) to obtain 8 subfractions (Fr. 5.1–5.8). Subfraction 5.4 (5.5 g) was further purified by Sephadex LH-20 column chromatography (PE : EtOAc : MeOH, 10 : 10 : 1) to give 6 subfractions (Fr. 5.5.1–5.5.6). Subfraction 5.5.4 (252 mg) was purified by semi-preparative RP-C18 HPLC (CH3CN/H2O, 70 : 30) to produce compounds 1 (7.2 mg) and 2 (6.5 mg). Above all, compounds 1 (7.2 mg) and 2 (6.5 mg) were obtained.

Compound characterization of 1 and 2
**Vlasoulide A (1).** White powder; [α]D25 = 1.00 (c 0.08, CH3OH); UV (CH3CN/H2O) λmax 210; IR (KBr) vmax 3438, 3079, 2933, 2869, 1770, 1710, 1639, 1448, 1382, 1351, 1268, 1222, 1153, 1272, 1020, 995, 896, cm−1; 1H and 13C-NMR data (600 MHz/150 MHz), see Table S1;† ESIMS m/z 561.4 ([M + Na]+); positive HRESIMS m/z 561.2828 ([M + Na]+), calcd 561.2823.

**Vlasoulide B (2).** White powder; [α]D25 = 0.00 (c 0.05, CH3OH); UV (CH3CN/H2O) λmax 210; IR (KBr) vmax 3404, 3079, 2929, 2869, 1772, 1752, 1639, 1448, 1400, 1382, 1353, 1282, 1257, 1224, 1068, 1018, 995, 896 cm−1; 1H and 13C-NMR data (600 MHz/150 MHz), see Table S1;† ESIMS m/z 561.5 ([M + Na]+); positive HRESIMS m/z 561.2829 ([M + Na]+), calcd 561.2823.

**(R)-and (S)-MTPA esters of compounds 1 and 2**
To each compounds 1 and 2 (each 1.5 mg) in pyridine-d5 (130 μL) was separately added (R)-(−)-MTPA (5 μL) and (S)-(+)-MTPA (5 μL) at room temperature, followed by stirring at 40 °C for 8 h, and each reaction mixture was transferred into a 1.7 mm NMR tube.

**(R)-MTPA ester of 1.** 1H NMR (pyridine-d5, 600 MHz): δH 1.20 (3H, d, H3-17), 2.88 (1H, m, H-15a), 2.91 (1H, m, H-15b), 4.30 (1H, t, H-6), 2.91 (1H, m, H-7), 2.21 (1H, m, H-8a), 1.58 (1H, m, H-8b).

**(S)-MTPA ester of 1.** 1H NMR (pyridine-d5, 600 MHz): δH 1.30 (3H, d, H3-17), 2.83 (1H, m, H-15a), 2.85 (1H, m, H-15b), 4.20 (1H, t, H-6), 2.88 (1H, m, H-7), 2.19 (1H, m, H-8a), 1.56 (1H, m, H-8b).

**(R)-MTPA ester of 2.** 1H NMR (pyridine-d5, 600 MHz): δH 1.30 (3H, d, H3-17), 2.71 (1H, m, H-15a), 2.74 (1H, m, H-15b), 4.21 (1H, t, H-6), 2.93 (1H, m, H-7), 2.14 (H, m, H-8a) 1.54 (1H, m, H-8b).

**(S)-MTPA ester of 2.** 1H NMR (pyridine-d5, 600 MHz): δH 1.22 (3H, d, H3-17), 2.77 (1H, m, H-15a), 2.78 (1H, m, H-15b), 4.27 (1H, t, H-6), 2.98 (1H, m, H-7), 2.16 (1H, m, H-8a), 1.55 (1H, m, H-8b).

Neuroprotection assay
The PC-12 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified eagle's medium (DMEM) containing 10% FBS supplemented with 100 μg mL−1 penicillin and 100 μg mL−1 streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C. Compounds 1 and 2 and vitamin E were dissolved in dimethyl sulfoxide (DMSO) and freshly prepared each time before use. Then, the PC-12 cells were seeded in 96-well culture plates at 8 × 104 cells per mL at 37 °C for 12 h. Then the cells were incubated with glutamate for an additional 24 h and the drugs (10, 15, 20 μM, respectively) were pretreated for 1 h before treated with glutamate. Cell viability was determined by the CCK-8 assay, after treatment, 10 μL of CCK-8 was added to each well and incubated at 37 °C for 4 h. The optical density (OD) was spectrophotometrically measured at 450 nm (CCK-8) using a microplate reader, respectively (BioTek Instruments, Inc.).

Results and discussion
Vlasoulide A (1) was isolated as white powder, possessing the molecular formula C12H14O7 determined by positive HRESIMS m/z 561.2828 ([M + Na]+), calcd 561.2823, indicating 12 degrees of unsaturation.

Comprehensive analysis of the 1H and 13C NMR spectra of 1, 32 carbon signals of the 13C NMR spectrum were classified by HSQC spectrum as one CH3, fourteen CH2 (including three sp2 carbons), nine CH (two oxygenated ones as well as one hydroxy methine), and eight quaternary carbons (assigned as two carbolnyl groups, two sp3 and six sp2 carbons respectively) (ESI, Tables S1†). Therefore, through a detailed analysis of 1D NMR spectra of 1 indicated that the compound 1 should be a dimeric sesquiterpene lactone.

The planar structure of 1 was determined by the analysis of 1H–1H COSY and HMBC spectra. Two proton-bearing structural fragments: H-5/H-6/H-7/H-8/H-9 and H-15/H-16/H-17 were observed by analyzing the 1H–1H COSY spectra, together with the HMBC correlations of H-13/C-1, C-9 and C-10; H-5/C-3 and C-12; H-7/C-14 and C-15 as depicted with arrows from H to C. The above conjectured that the unit A should be deduced as a ring-opening guaianolide moiety. Furthermore, a five-membered ring newly generated fused with the seven-
membered ring and the lactone ring in unit A at C-1, C-4 and C-5 which revealed by the $^1$H–$^1$H COSY correlations of H$_2$-3/H$_2$-2/H-1/H-5 and the HMBC cross-peaks from H-5 ($\delta_H$ 2.78) to C-3 and C-4 (Fig. 2). Meanwhile, the structure of unit B was further determined to be a guaianolide moiety, confirmed by $^1$H–$^1$H COSY correlations of H$_2$-3’/H$_2$-2’/H-1’/H-5’/H-6’/H-7’/H$_2$-8’/H$_2$-9’ as well as the HMBC correlations of H$_2$-14’/C-1’, C-9’ and C-10’; H$_2$-15’/C-3’, C-4’ and C-5’; H$_2$-13’/C-7’, C-11’ and C-12’ (Fig. 2). Finally, the units A and B were linked directly via a C–C bond between C-13’ and C-12 according to the $^1$H–$^1$H COSY correlations of H$_2$-13’/H$_2$-12, combine with the HMBC correlations of H$_2$-13’ ($\delta_H$ 1.81, 1.69, 1H, respectively)/C-12 ($\delta_C$ 30.5), C-4 ($\delta_C$ 54.3), C-7’ ($\delta_C$ 48.7), C-11’ ($\delta_C$ 76.7) and C-12’ ($\delta_C$ 176.8). Thus, the planar structure of 1 was determined as shown in Fig. 1.

The relative configuration of 1 was established by the NOESY spectrum. H-1, H-5, H-7 and H-1’, H-5’, H-7’ in units A and B were on the same face and assigned as $\alpha$-orientation and $\beta$-orientation in 1 based on the similar NOESY correlations of H-1/H-5/H-7 and H-1’/H-5’/H-7’. And, the H-6/H-7 and H-6’/H-7’ were in the trans-form due to the large coupling constant between H-6/H-7 ($J = 9.8$ Hz) and H-6’/H-7’ ($J = 9.5$ Hz) (Fig. 2). In addition, the relative configuration of C-11’ and C-4 were resolved by the NOESY correlations of H-6’/H-13’ and H$_2$-12/H-1/H-5 (Fig. 2), exhibiting that the CH$_2$-13’ and CH$_2$-12 were $\alpha$-oriented. At the same time, by comparing the calculated and experimental ECD spectra, we found that the experimental ECD spectrum of compound 1 (Fig. 3A) was in good accordance with the calculated curve for the (1S,4R,5S,6S,7S,16R,1’R,5’R,6’R,7’R,11’S) stereoisomer. Additionally, the observed $\Delta\delta_{(R,S)}$ values of the (S)- and (R)-MTPA esters established the absolute configuration of C-16 in 1 as $R^R$ (Fig. 4), which was consistent with the ECD results. Thus, the structure and absolute configuration of the vlasoulide A (1) was fully defined as shown in Fig. 1.

Interestingly, vlasoulide B (2), a 16-epimer of 1, was also an optically active white powder. HRESIMS data at m/z: 561.2829 ([M + Na]$^+$, calcld 561.2823) of 2 implied that its molecular formula was C$_{32}$H$_{42}$O$_{7}$, same as 1. The $^1$H and $^{13}$C NMR data of 2 resembled those of compound 1 (ESI, Tables S1), suggesting the presence of ring-opening guaianolide and guaianolide units. The analysis of its 2D NMR data ($^1$H–$^1$H COSY and HMBC) revealed that the two compounds had the same 2D structure. Furthermore, inspecting the differences between 1 and 2 on NMR data we found the most obvious change was the C-15 and H$_2$-15, the chemical shift of C-15 was downfield shifted from $\delta_C$ 50.5 in 1 to $\delta_C$ 51.6 in 2, meanwhile, an obvious up field shifted of H$_2$-15 from $\delta_H$ 2.67, 2.66 in 1 to $\delta_H$ 2.63, 2.62 in the $^1$H NMR was observed. Thus, these two new compounds represent a pair of stereoisomers possessing opposite configuration at C-16, which was identical to the $\Delta\delta_{(R,S)}$ Results (Fig. 4). At the same time, the experimental ECD spectrum of 2 also fits well with the calculated spectrum of (1S,4R,5S,6S,7S,16S,1’R,5’R,6’R,7’R,11’S) 2 (Fig. 3B). Therefore, the structure of vlasoulide B (2) was established as shown in Fig. 1.

All the isolated compounds were evaluated for their neuroprotective effects against glutamate-induced neurotoxicity in PC-12 cells using CCK8 assay. Compound 1 showed prominent neuroprotective activity against glutamate-induced neurotoxicity in PC-12 cells, with EC$_{50}$ value of 13.54 ± 0.33 µM. While, the EC$_{50}$ values of the compound 2 is greater than 30 µM.
Meanwhile, the neuroprotective effects were dose dependent and there were no significant adverse effects on the growth of PC-12 cells with compound 1 solo treatment as shown in Fig. 5. Collectively, these results suggest that compound 1 showed significant neuroprotective activity against glutamate-induced neurotoxicity in PC-12 cells at concentration of 20 μM.

Conclusions

In conclusion, vlasoulides A and B (1 and 2), two rare and original C32 sesquiterpenoid lactone dimers comprising of two sesquiterpene lactone units, have been isolated and elucidated for the first time from Vladimiria souliei. Moreover, compound 1 showed neuroprotective activity against glutamate-induced neurotoxicity in PC-12 cells, with EC50 value of 13.54 ± 0.33 μM. These results will supply scientific foundation for the scientific research of this plant, as well as might be greatly useful for studying on neuroprotection.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

1 Editorial commission of Traditional Chinese Medicine, State Administration of Traditional Chinese Medicine, *Traditional Chinese Medicine*, Shanghai Science & Technology Press, 1999, vol. 7, pp. 815–817.
2 J. J. Chen, D. Q. Fei, S. G. Chen and K. Gao, *J. Nat. Prod.*, 2007, 18, 547–550.
3 J. J. Chen, H. B. Wei, Y. Z. Xu, J. Zeng and K. Gao, *Planta Med.*, 2013, 79, 1470–1473.
4 Q. H. Wu, C. M. Liu, Y. J. Chen and K. Gao, *Helv. Chim. Acta*, 2006, 89, 915–922.
5 J. Xu, X. J. Zhao, Y. Q. Guo, W. B. Hou and S. Z. Zhang, *Chin. Chem. Lett.*, 2009, 20, 1472–1474.
6 C. L. Li and J. Sheng, *Adv. Mater. Res.*, 2013, 634, 901–904.
7 Z. G. Wang, X. R. Lan, Y. Y. Xiao and Y. Zhang, *Chin. Pharm.*, 2006, 17, 303–304.
8 J. J. Chen, W. Bai, F. R. Gobu, C. H. Wu, J. Zeng and M. Sun, *J. Asian Nat. Prod. Res.*, 2015, 17, 1–8.
9 J. Xu, P. Zhang, Z. J. Ma, Y. Q. Guo, X. J. Zhao and K. Wei, *Phytochem. Lett.*, 2009, 2, 204–206.
10 Y. X. Yang, S. Gao and H. L. Li, *RSC Adv.*, 2015, 7, 31–40.
11 R. X. Tan, J. Jakupovic, F. Bohlmann and Z. J. Jia Schuster, *Phytochemistry*, 1990, 29, 1209–1212.
12 L. P. Chen, G. Z. Wu, J. P. Zhang and H. L. Li, *Sci. Rep.*, 2017, 43837.
13 Z. L. Wu, J. X. Wang, L. P. Chen, H. L. Li and W. D. Zhang, *Fitoterapia*, 2018, 125, 117–122.
14 H. Wei, G. Ma, Y. Peng, C. He and P. Xiao, Chemical Constituents of the Roots of Dolomiaea souliei, *Chem. Nat. Compd.*, 2014, 37, 1249–1253.
15 Z. L. Wu, Q. Wang, H. Y. Dong, H. L. Li and W. D. Zhang, *Fitoterapia*, 2018, 128, 192–197.
16 Z. L. Wu, Q. Wang, J. X. Wang, X. K. Xu, Y. H. Shen, H. L. Li and W. D. Zhang, *Org. Lett.*, 2018, 20, 7567–7570.
17 H. Zhang, L. T. Xu, D. M. Ren, et al., *Chin. Chem. Lett.*, 2020, 31, 1259–1262.